Structure-conformation-activity studies of the melanin concentrating hormone (MCH)

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STRUCTURE-CONFORMATION-ACTIVITY STUDIES OF THE MELANIN CONCENTRATING HORMONE (MCH)

submitted by CHERYL ANNE MOSS

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To my Parents

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Abstract

The first part of this thesis describes a "Stage 1" study involving systematic investigation of relationships between structure, conformation and activity of the Melanin Concentrating Hormone (MCH). The parent peptide and a series of sub-structural fragments have been prepared by solid phase synthesis and their biological potencies compared. It is proposed that the exocyclic sequences of MCH may assist positioning/binding of the molecule to achieve maximal receptor site interaction. The disulphide linkage is essential to the maintenance of activity, promoting the active conformation.

As the first stage in elucidating the shape and charge characteristics of MCH, the linear and cyclic 5-14 sub-structural units were used as models in ^{1}H n.m.r. conformational studies. In addition, molecular dynamics analyses were performed independently on the cyclic 5-14 fragment and the parent peptide. The theoretical studies indicate that two major families of conformations may be accessed, determined by flexibility at Gly^{8}. Conformational features predicted by molecular dynamics find support in the ^{1}H n.m.r. studies. A common predominant feature is a transannular hydrogen bond from the Tyr^{11} side chain, suggesting its importance in stabilising the biologically active conformation.

In the second part of this thesis, the important structural and conformational features revealed by the initial analyses were investigated further in the "Stage 2" study. Analogues of the cyclic 5-14 subunit were prepared comprising substitutions at Gly^{8} and Tyr^{11}, the former resulting in a definable conformational constraint. Subsequent biological testing and conformational analyses confirm the importance of the substituted residues for activity, and indicate the possible geometry of Gly^{8} in the active conformation.

The third section of this thesis is concerned with synthetic approaches towards a suitable disulphide mimic, based on the allenyl *bis*-amino acids. Such a surrogate is required to allow further investigation of the role of cystine in promoting the biologically active conformation of MCH.

Abbreviations

Ac	acetyl
Acm	acetamidomethyl
ACTH	adrenocorticotropin
Adoc	adamantyloxycarbonyl
Aha	7-aminoheptanoic acid
Aib	α-aminoisobutyric acid
a.m.u.	atomic mass unit
t-BDPSi	*tert*-butyldiphenylsilyl
Boc	*tert*-butyloxycarbonyl
Bom	benzyloxymethyl
Bop	benzotriazolyloxy*tris*(dimethylamino)-phosphonium hexafluorophosphate
b.p.	boiling point
Bpoc	2-(*p*-biphenylyl)propyl(2)oxycarbonyl
t-Bu	*tert*-butyl
Bum	*tert*-butoxymethyl
Bzl	benzyl
C.I.	chemical ionisation
COSY	correlation spectroscopy
DCC	*N,N'*-dicyclohexylcarbodiimide
DCM	dichloromethane
DCU	*N,N'*-dicyclohexylurea
DEPC	diethyl phosphorocyanidate
DFAM	diethyl formylaminomalonate
Dhbt	3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine
Dibal-H	diisobutylaluminium hydride
DIPCDI	*N,N'*-diisopropylcarbodiimide
DMAP	4-*N,N*-dimethylaminopyridine
DMF	*N,N*-dimethylformamide
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DPPA	diphenylphosphoryl azide
Dts	dithiasuccinoyl
EDT	ethanedithiol
E.I.	electron impact
Et	ethyl

F.A.B.	fast atom bombardment
For	formyl
Fmoc	9-fluorenylmethyloxycarbonyl
h	hour
HOBt	1-hydroxybenzotriazole
Hz	Hertz
i.r.	infrared
J	coupling constant
LRCOSY	long range correlation spectroscopy
M	molar
MBHA	4-methylbenzhydrylamine (resin)
MCH	melanin concentrating hormone
Me	methyl
mequiv.	milliequivalent
mg	milligram
min	minute
mmol	millimole
m.p.	melting point
mRNA	messenger ribonucleic acid
m.s.	mass spectrum
Ms	mesyl (methanesulphonyl)
MSH	melanin stimulating hormone
Mtr	4-methoxy-2,3,6-trimethylbenzenesulphonyl
m/z	mass to charge ratio
N	normal
n.m.r.	nuclear magnetic resonance
NOE	nuclear Overhauser effect
NOESY	2D NOE
Nps	*o*-nitrophenylsuphenyl
O-*t*-Bu	*tert*-butyl ester
ONb	*o*-nitrobenzyl
(P)	polymeric resin support
PAM	phenylacetamidomethyl
Pfp	pentafluorophenyl
Ph	phenyl
PMC	2,2,5,7,8-pentamethylchroman-6-sulphonyl
POMC	pro-opiomelanocortin

ppm	parts per million
R_f	retention index for thin layer chromatography
ROESY	rotating-frame NOESY
RPHPLC	reverse phase high performance liquid chromatography
RT	room temperature
TEA	triethylamine
TFA	trifluoroacetic acid
TFMSA	trifluoromethanesulphonic acid
THF	tetrahydrofuran
t.l.c.	thin layer chromatography
TMSOTf	trimethylsilyl trifluoromethanesulphonate
TNBSA	2,4,6-trinitrobenzenesulphonic acid
t_R	retention time for HPLC
Trt	trityl
Ts	tosyl (*p*-toluenesulphonyl)
u.v.	ultraviolet
v/v	volume per unit volume
w/v	weight per unit volume

Commonly Occurring L-Amino Acids

Ala	alanine
Arg	arginine
Asn	asparagine
Asp	aspartic acid
Cys	cysteine
Gln	glutamine
Glu	glutamic acid
Gly	glycine
His	histidine
Ile	isoleucine
Leu	leucine
Lys	lysine
Met	methionine
Nle	norleucine
Phe	phenylalanine
Pro	proline
Ser	serine
Thr	threonine
Trp	tryptophan
Tyr	tyrosine
Val	valine

Nomenclature

Abbreviations used for amino acids and the designation of peptides follow the rules of the IUPAC - IUB Commission on Biochemical Nomenclature.[1] For clarity, structures are numbered sequentially, from Chapter 1 onwards, in brackets and emphasised type. References are shown in superscripted, emphasised type.

The symbol *, is given at the beginning and the end of sections which describe the work of colleagues, (see Appendix), which is included for completeness.

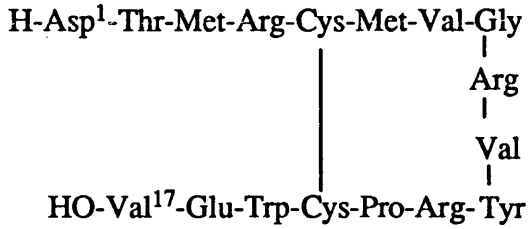
CONTENTS

INTRODUCTION

Chapter 1

INTRODUCTION

Melanin concentrating hormone (MCH) (**1**) is a neuropeptide which is



(1)

involved in controlling body colour in lower vertebrates.[2] In teleost fish, it serves to concentrate melanin within the pigment cells of the skin, causing lightening. However, MCH is also known to induce melanosome dispersion within tetrapod melanophores.[3] In addition, it acts as a potent pituitary regulating hormone, inhibiting the release of adrenocorticotropin (ACTH) in fish and mammals,[4] and stimulating the release of growth hormone in rats.[5]

MCH has been isolated from several sources,[6,7a,8] and its primary structure elucidated[7] as a cyclic heptadecapeptide, with a disulphide-bridged ring sequence and N- and C- terminal exocyclic residues. Syntheses of MCH,[9-11] and various analogues,[8,12-14] have been carried out by different groups. Determination of their biological activities has shown that the cyclic ring is essential.[8,12]

It is evident that conflicting data have also been published and that the topological and charge distribution properties of the cyclic system have not been considered until very recently.[15] Knowledge of structural and conformational characteristics is essential to the understanding of determinants for binding and activity. The object of this research programme was to investigate these features and our findings are described.

Since the majority of the project work was concerned with solid phase peptide synthesis of MCH analogues required for our study, it is pertinent to review the salient features of this methodology. Consideration is also given to techniques which may be applied in the determination of relationships between peptide structure, conformation and activity. In addition, the biological properties of MCH as a potent

pituitary regulating hormone are examined.

1.1. SOLID PHASE PEPTIDE SYNTHESIS

Peptides[16] and proteins exhibit some of the largest structural and functional variation of all classes of biologically active molecules. They are constructed from the twenty genetically coded L-amino acids, and other moieties, and sequences may be linear, cyclic, or branched.

It has been recognised that peptides comprising 5-50 amino acids are involved in many physiological processes. They may be released from the endocrine glands into the blood which carries them to the site of action, or be specialised neurotransmitters.

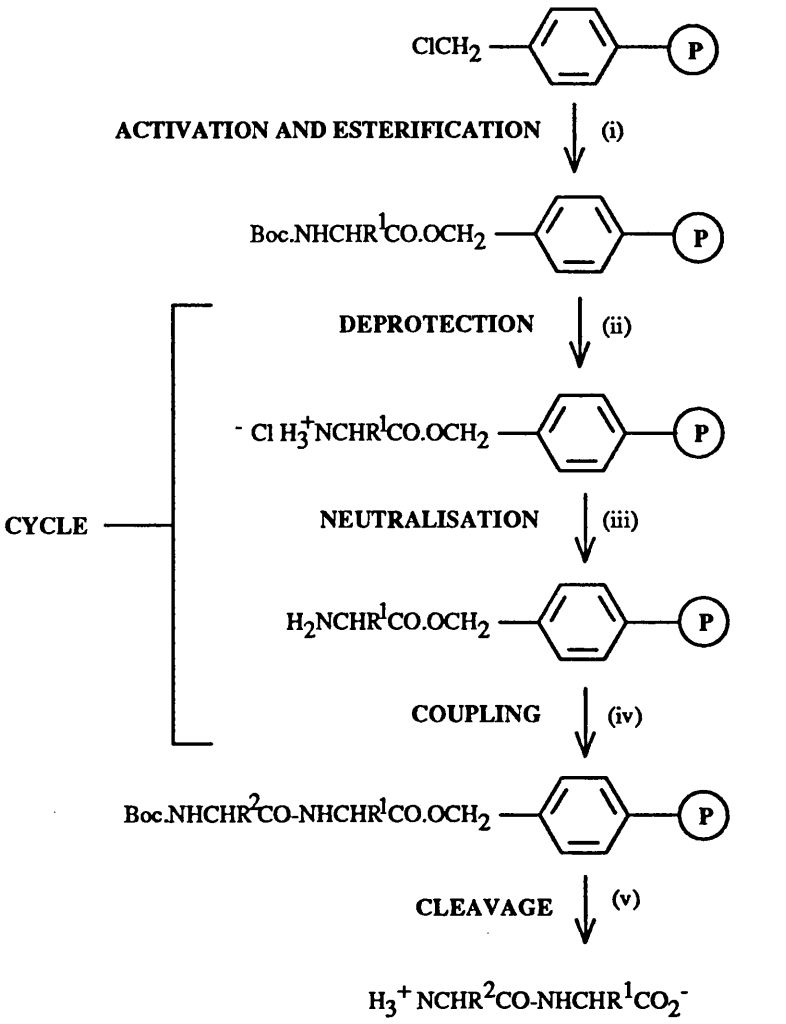
Synthesis can not only confirm the structures of naturally occurring peptides but also provide large quantities for further investigation. Thus, a rapid, efficient and reliable methodology is required for such purposes. The "classical" solution phase approach to peptide synthesis has yielded impressive successes in the preparation of biologically active peptides.[17] Chain elongation may proceed via incremental or segment condensation processes. However, these procedures are not ideally suited to the preparation of long chain peptides. In recognition of problems inherent in the solution phase method, the search for a more efficient procedure resulted in the development of the solid phase approach.[18]

Solid phase peptide synthesis, first described by Merrifield,[19] has proved to be an extremely efficient procedure for the chemical synthesis of peptides. The attachment of a growing peptide chain to a solid support has provided an element of speed and efficiency, lacking in the "classical" approach. The feasibility of this procedure for both manual and automated preparation has also been demonstrated. Indeed, the method has been successfully applied to the syntheses of other oligomeric structures, in particular DNA.[20]

1.1.1. The Solid Phase Principle

The essential elements of a solid phase synthesis, typical of the standard Merrifield approach, are outlined in **Scheme 1.**

Attachment of the first amino acid is effected by a covalent bond to the



(i) Boc-amino acid , EtOH , 80°C (ii) HCl-AcOH or TFA-DCM

(iii) TEA-DCM (iv) Boc-amino acid , DCC , DCM (v) HBr -TFA or HF

Scheme 1

solid polymeric support. It is necessary to block temporarily the amine function of the incoming amino acid, otherwise, once activated a self-condensation reaction would occur. Side chains of certain amino acid functionalities may also need protection that is maintained throughout the synthesis. Once esterification of the first amino acid is complete, the temporary N^{α}-protecting group is removed. When acid is used for this deprotection, a neutralisation step is required to release the free amine. The synthesis then continues with repetitive cycles of acylation and deprotection, adding the succeeding amino acids one at a time in a stepwise manner until the desired sequence has been assembled. Reactions are

required to proceed in extremely high yields since there is no purification of intermediates. Excess soluble reagent can be employed to drive reactions to completion and may be readily removed by simple filtration and washing.

Once the synthesis is complete, the peptide is released from the support using conditions that are least destructive toward sensitive residues. Finally, scrupulous purification and characterisation is required so as to ensure the absolute integrity of the product.

Many improvements and variations of the general procedure outlined in **Scheme 1** have developed in recent years, leading to increasingly sophisticated methodologies, adapted to suit the objectives of the synthetic endeavour.

1.1.2. Sequence Assembly Methods

Most commonly, stepwise condensation is employed where chain growth proceeds from the C- to N-terminal direction. The reverse is less favourable due to the concomitant racemisation. Convergent solid phase synthesis, involving coupling of protected peptide segments on the solid support, has also been successfully applied. This induces an overall simplication of the synthesis and is particularly appropriate for the preparation of longer sequences. However, difficulties may arise through low solubility of intermediates, rearrangements and racemisation. Anchoring may also be carried out via an amino acid side chain, or both components to be coupled can be anchored on separate supports, enabling amino and carboxyl components to be removed in separate steps.

1.1.3. Solid Supports and Reaction Media

Commonly, cross-linked polystyrene or polyamide polymers are used forming microporous gels, where sites are uniformly distributed throughout the polymeric matrix. The solvents of choice are generally DCM and DMF, in which the dry beads swell, forming a well solvated gel where the peptide chains are mobile and reagent-accessible, enabling adequate reaction rates to be attained. A new mixed solvent, N-methylpyrrolidone-dimethylsulphoxide has shown improved resin solvation properties and reduction of peptide chain aggregations.[21]

Other non-swellable supports include a range of carbohydrates, macroporous polystyrenes and polyethylenes, and "controlled-pore" silica glass. Recent innovations have been the introduction of polypropylene membranes[22] and cellulose disc supports.[23]

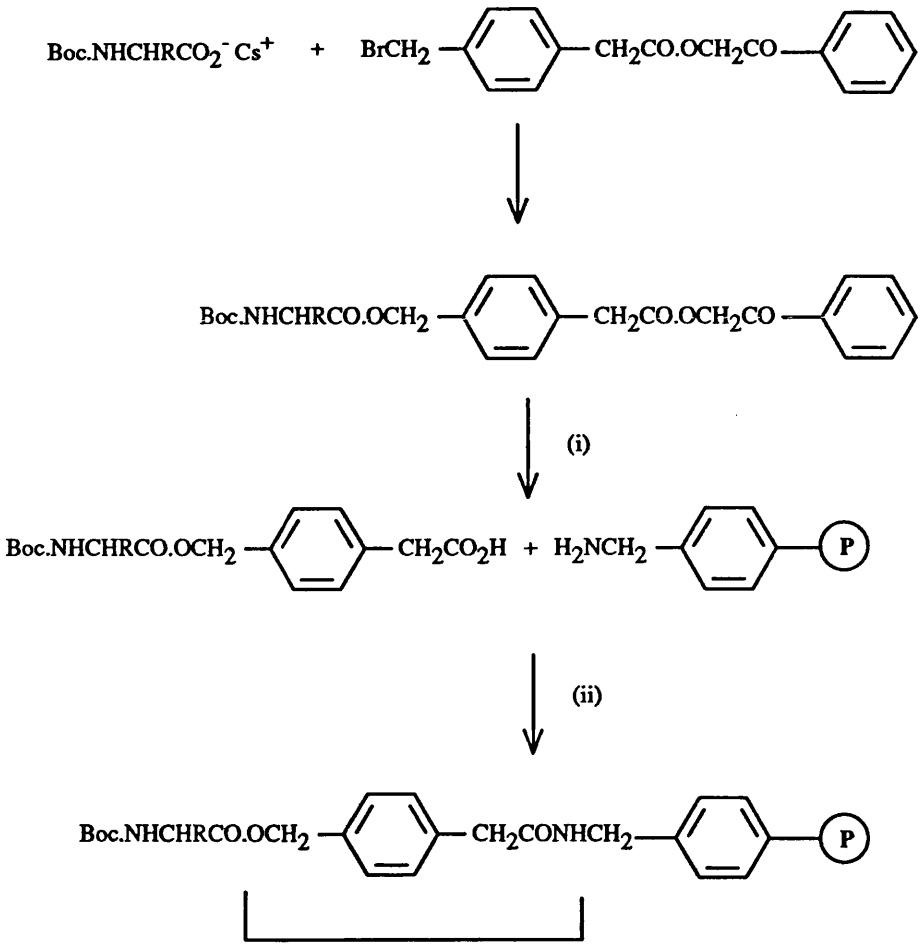
In continuous-flow processes,[24] where reagents and solvents are pumped through column-packed resins, the simple gel media may be unsatisfactory, lacking the necessary rigidity and strength. Suitable supports are obtained by enclosing the gel in a rigid macroporous framework, such as kieselguhr.

1.1.4. Functionalisation and First Residue Attachment

The polymeric support must contain the appropriate functional groups onto which the first residue is anchored. The choice of functional group is determined by the nature of the peptide product required and compatibility with the overall protection strategy adopted. The linkage must remain stable under conditions required to effect cleavage of the temporary N^{α}-protecting group.

A multitude of functionalisation methods have been described.[18] The first N^{α}-protected amino acid can be linked directly to a suitably functionalised resin. More recently, however, the quantitative introduction of the preformed handle derivative has proved to be particularly valuable. In general, a handle is defined as a bifunctional spacer that incorporates the features of the desired C-terminal group and serves to link the initial synthetic unit of the peptide chain to a previously functionalised solid support in two discrete steps. The most versatile strategy, illustrated in **Scheme 2**, for the phenylacetamidomethyl (PAM) handle, involves formation of the bond to the synthetic unit first. This allows purification and characterisation, before quantitative attachment to the support via an amide linkage. Therefore, this allows complete control over the final loading of the initial amino acid, and circumvents potential problems associated with extraneous functional groups on the resin.

A range of "safety-catch" or "multi-detachable"[25] anchors may also be employed which are respectively cleaved in more than one step, or at more than one site, thus allowing formation of protected peptide segments or



Scheme 2

cyclic peptides.

Selected examples of various anchoring linkages and handles are given in **Figures 1 and 2**, showing compatible N^{α}-protecting groups and cleavage conditions. It is therefore evident, that on cleavage, the choice of anchoring linkage can determine both the extent of side-chain protection maintained and the nature of the C-terminal functionality.

There are various methods available for acylation of the first amino acid to the functionalised polymer. A common methodology, when using chloromethyl-resins, involves reaction with the N^{α}-protected C-terminal amino acid as its triethylammonium or caesium salt. For benzyl alcohol-resins, the N^{α}-protected C-terminal amino acid may be coupled by

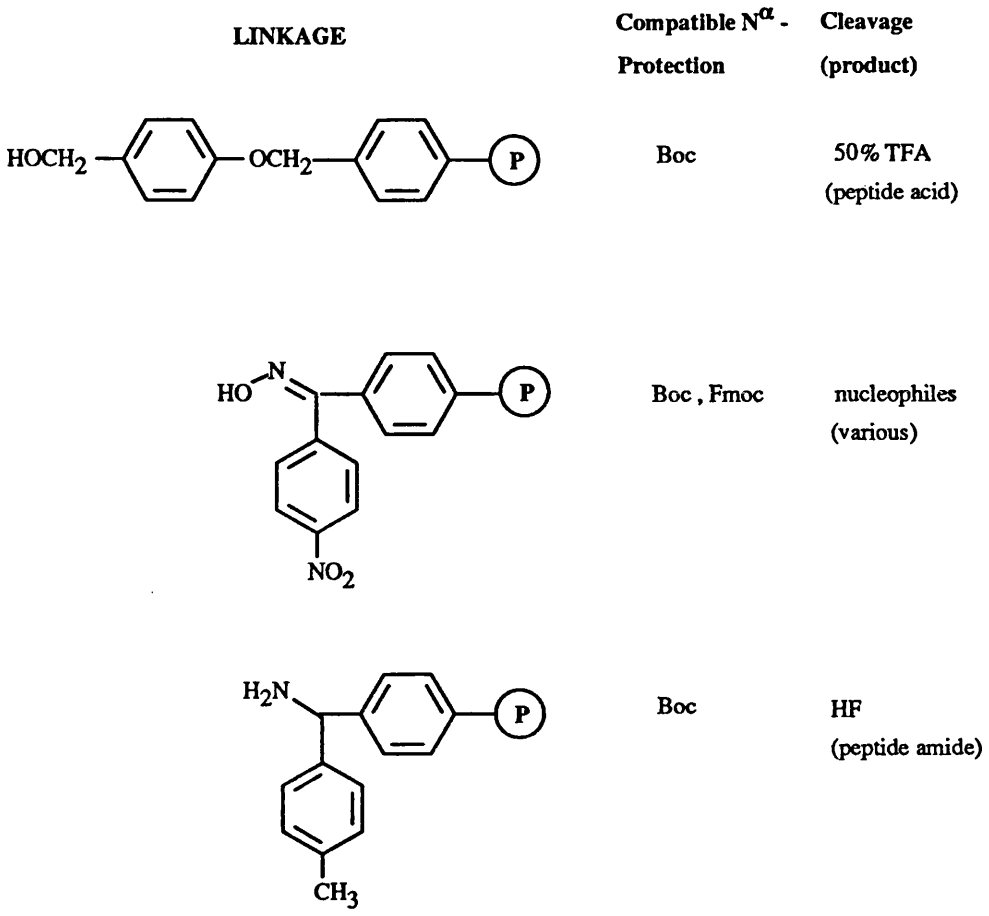
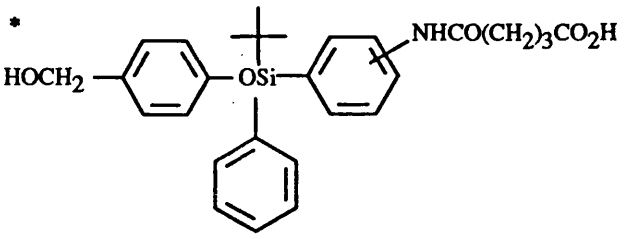
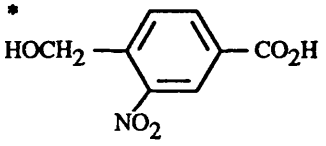
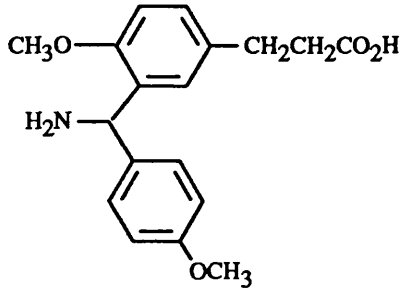


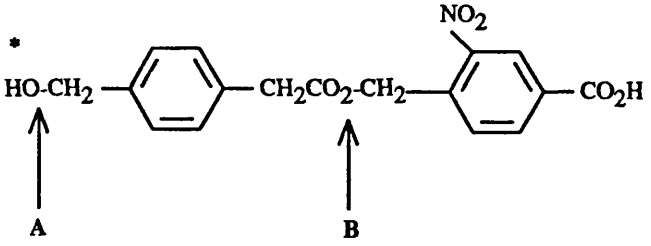
Figure 1

the use of *N,N'*-dicyclohexylcarbodiimide (DCC) in the presence of 4-*N,N*-dimethylaminopyridine (DMAP) as a catalyst.[26] Conditions have been established for minimising DMAP promoted racemisation in this step.[27] Cleavage of N^{α}-Fmoc by DMAP during the esterification reaction was considered a potential hazard but does not appear to be significant. Alternative methods are available, avoiding the use of DMAP, including esterification of Fmoc-amino acids to benzyl alcohol-resins by using 2,6-dichlorobenzoyl chloride.[28]

For coupling reactions of peptide segments on the solid support, protected peptide fragments may be esterified through the C-terminus, or a free side-chain carboxyl group, onto a suitably functionalised resin.

HANDLE	Compatible N^{α}-Protection	Cleavage (product)
	Fmoc , Dts	$(n\text{-Bu})_4 N^+F^-$ (peptide acid)
	Fmoc , Dts	hv : 350 nm (peptide acid)
	Fmoc	TFA (peptide amide)

MULTI-DETACHABLE HANDLE



	Boc	**A :** HF (peptide acid) **B :** hv : 350 nm (peptide handle)

* partially protected segments may be obtained following cleavage

Figure 2

1.1.5. General Protection Schemes

The choice and optimisation of the general protection scheme is crucial to the success of any peptide synthesis. The temporary N^{α}-protecting groups must survive during each coupling step, yet removal must be rapid, quantitative and free from any side reactions.

Protecting groups must also be employed for the side chains of certain amino acids to prevent branching or other undesired reactions. It is critical that they are stable to the repeated cleavage of the N^{α}-protecting group, yet be readily removed when desired, with minimum side reactions.

There are two principal methods of solid phase synthesis in popular use today, based on different general protection schemes. These are the standard Boc-polystyrene and the recently developed Fmoc-polyamide[29] methodologies (nomenclature derived from the N^{α}-protecting group and the nature of the solid support). The Boc-polystyrene procedure is more akin to the original Merrifield methodology,[19] as outlined in **Scheme 1**, yet the basis of each is founded on the same principles with differing specific chemistries.

1.1.5.1. Boc-Polystyrene Procedure

Commonly, the resin used is a polystyrene suspension polymer cross-linked with 1% of *m*-divinyl benzene. Reactions are usually performed in DCM, though favourable results have been obtained with a polystyrene-DMF combination.[30] Differential cleavage is achieved by use of protecting groups with graduated acid lability. Temporary N^{α}-protection is effected by employing *t*-butyloxycarbonyl (Boc) derivatives, typically cleaved by trifluoroacetic acid (TFA). The adamantyloxycarbonyl (Adoc) group has also found application. Although offering no intrinsic advantage over the Boc group, this derivative may be preferred in certain circumstances because of its superior solubility characteristics. Various benzyl ethers, esters and urethane derivatives with electron withdrawing substitutents, for greater acid stability, are generally employed for protection of hydroxyl and carboxyl based amino acid side chains. Alternatively, ether and ester derivatives based on cyclopentyl and cyclohexyl alcohols have also been used in attempts to negate certain side reactions associated with the benzyl substitutents, such as C-alkylation and

$\alpha \rightarrow \beta$ rearrangements. The side-chain protecting groups require more vigorous conditions for removal, and anhydrous liquid hydrogen fluoride (HF) is often the reagent of choice.

A typical Boc-polystyrene protection scheme is outlined in **Figure 3.**

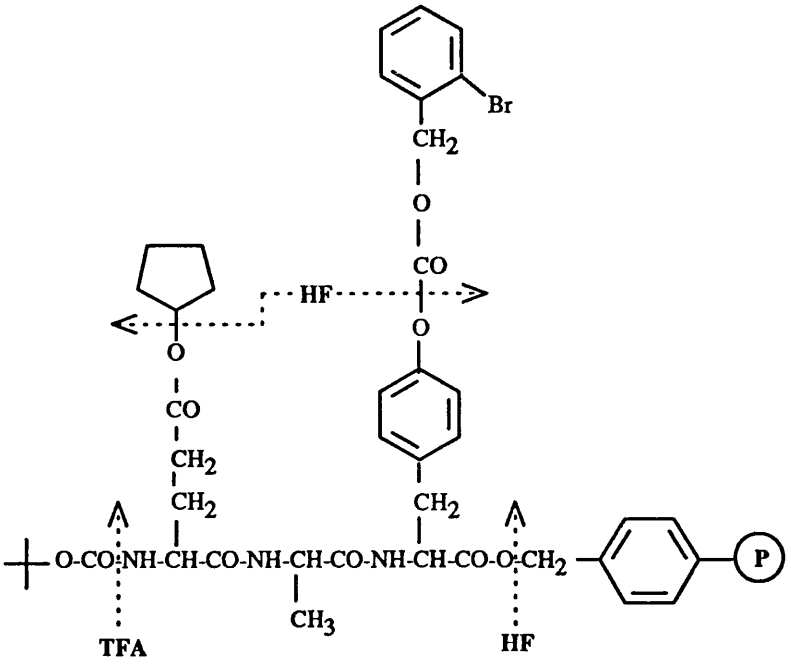


Figure 3

1.1.5.2. Fmoc-Polyamide Procedure

This method was developed particularly as a consequence of concern arising from published accounts of many side reactions resulting from repetitive strong acid treatment used in the Boc-polystyrene procedure.[31]

In the Fmoc-polyamide method, the base labile 9-fluorenylmethyloxycarbonyl (Fmoc)[32] group is used for temporary α-amino protection. It is cleaved within seconds, via β-elimination, by secondary amines such as piperidine, which simultaneously scavenge the dibenzofulvene intermediate. The use of Fmoc enables the adoption of *t*-butyl based side-chain protection, cleaved under relatively moderate conditions by TFA, as opposed to more acid-stable benzyl based derivatives used in the Boc-polystyrene procedure. This permits the solid phase synthesis to be carried out under considerably milder conditions.

Beaded polar polydimethylacrylamide is used as the solid support. It is freely solvated by a wide range of solvents but particularly by dipolar aprotic media such as DMF. This leads to an apparent reduction of peptide aggregation which can occur within the resin matrix and provides a particularly favourable reaction medium for both acylation and deprotection reactions.

A general protection scheme for the Fmoc-polyamide methodology is shown in **Figure 4.**

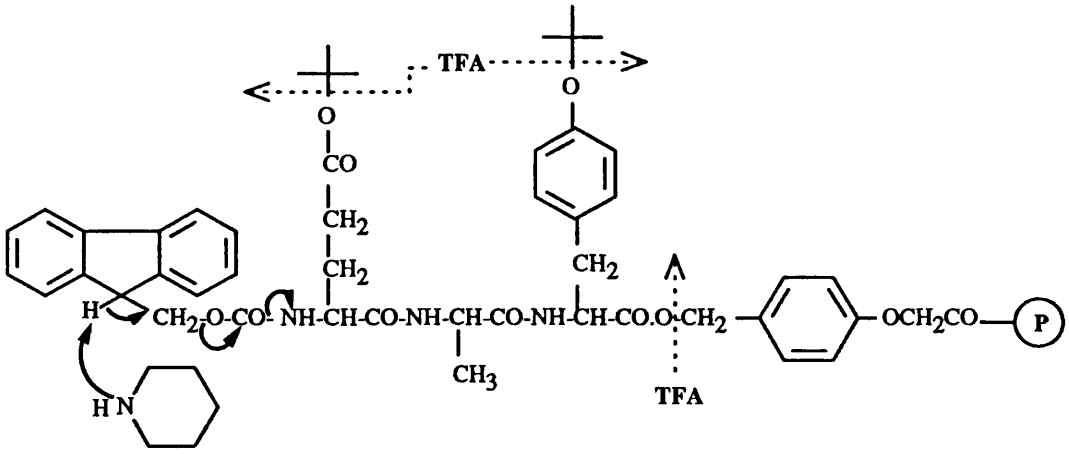


Figure 4

Other mild protection schemes may employ dithiasuccinoyl (Dts), 2-(*p*-biphenylyl)propyl(2)oxycarbonyl (Bpoc) or *o*-nitrophenylsulphenyl (Nps) as alternative N^{α}-protecting groups, cleaved by reduction, very mild acidolysis and nucleophilic attack respectively. Indeed the Dts group has been applied in a three dimensional orthogonal protection scheme.[33] Since its cleavage may be effected by treatment with thiols, the use of *t*-Bu based side-chain protection was employed, while the third dimension was introduced by the *o*-nitrobenzyl (ONb) ester anchoring linkage, cleaved by photolysis. Any one of the cleavages could be performed without affecting the other classes, therefore enabling the synthesis of a range of partially protected or fully deblocked peptides.

Although general protection schemes have been outlined, certain bifunctional amino acids require more specialised protection. Some selected examples are given in **Figures 5 and 6** which show compatibility with either the Boc-polystyrene or Fmoc-polyamide methodologies.

Compatible With The Boc-Polystyrene Procedure :

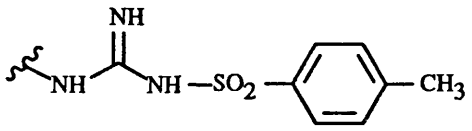
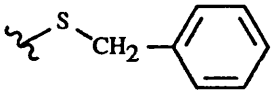
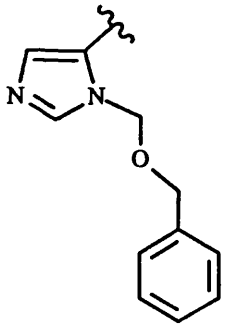
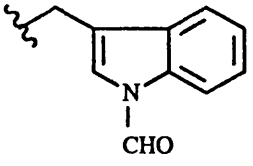
Partial Structure	Stability	Removal
 Arg(Tos)	HBr ; TFA	HF
 Cys(Bzl)	HF , 0°C	HF , 25°C ; Na / liquid NH_3
 His(Bom)	base ; TFA	HBr - AcOH , 25°C ; HF , 0°C ; cat . H_2
 Trp(For)	HF	aq . pH 12 , 25°C ; HF - Me_2S

Figure 5

Some protecting groups are also compatible with both procedures, and are removed by methods orthogonal to acidolysis. Some selected examples are given in **Figure 7.**

Both the Boc-polystyrene and Fmoc-polyamide procedures have had many successful applications. The associated mildness of the Fmoc-polyamide methodology may be advantageous in syntheses of sequences that are particularly sensitive to acid.

Compatible With The Fmoc-Polyamide Procedure :

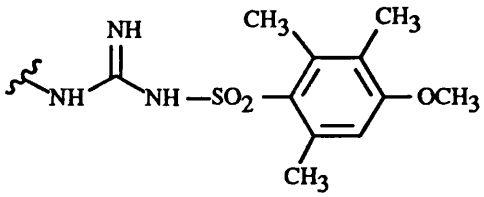
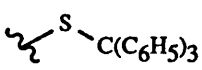
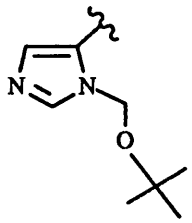
Partial Structure	Stability	Removal
 Arg(Mtr)	piperidine - DMF ; dilute TFA	TFA - thioanisole , 25°C
 Cys(Trt)	base	Hg^{2+} ; Ag^{+} ; I_2 ; TFA - anisole , 25°C
 His(Bum)	base	TFA , 25°C

Figure 6

Compatible With Boc-Polystyrene And Fmoc-Polyamide Procedures :

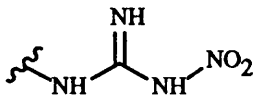
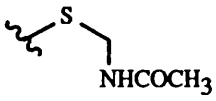
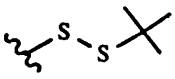
Partial Structure	Stability	Removal
 Arg(NO_2)	HBr ;TFA	HF , 25°C ; cat. H_2
 Cys(Acm)	HF	Hg^{2+} ; I_2
 Cys(S-*t*-Bu)	HF	RSH or other reducing agents

Figure 7

1.1.6. Coupling Methodologies

Various coupling techniques may be employed to effect elaboration of the peptide chain once the first-residue attachment is complete.

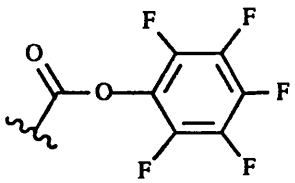
Use of *in situ* condensing agents is a commonly employed methodology for the activation of the carboxyl group in the presence of the free amine. The most popular reagents continue to be the carbodiimides of which DCC[34] has been most widely applied. Occasionally, *N,N'*-diisopropylcarbodiimide (DIPCDI)[35] has been used since it gives rise to more soluble urea by-products. Frequently, 1-hydroxybenzotriazole (HOBt)[36] is also added in the presence of the condensing agent. It has been found that HOBt is an extremely efficient catalyst in peptide bond forming reactions that also acts to suppress racemisation. Most likely, formation of the HOBt ester of the incoming N^{α}-protected amino acid yields an extremely efficient acylating agent which reacts without racemisation.

Symmetrical amino acid anhydrides are also popular, highly reactive derivatives. They are usually prepared just prior to use from the

corresponding N^{α}-protected amino acid and DCC in DCM.

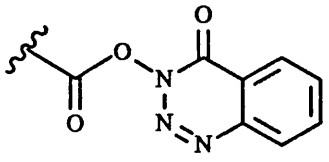
Mixed anhydrides have also been applied, but their use has been limited due to low regiospecificity in aminolysis of the anhydride. However, phosphinic carboxylic mixed anhydrides are potentially useful.[37]

Reliable reagents of choice continue to be active esters. The esters of *N*-hydroxysuccinimide have proved to be effective in solid phase syntheses. The *p*-nitrophenyl and trichlorophenyl derivatives have also been used but reaction rates are relatively slow. However, pentafluorophenyl (Pfp)[38] (2) and 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine (Dhbt)[39] (3) esters have been found to be efficient acylating agents. The reactivity of the former is enhanced by added HOBt.



Pfp ester

(2)

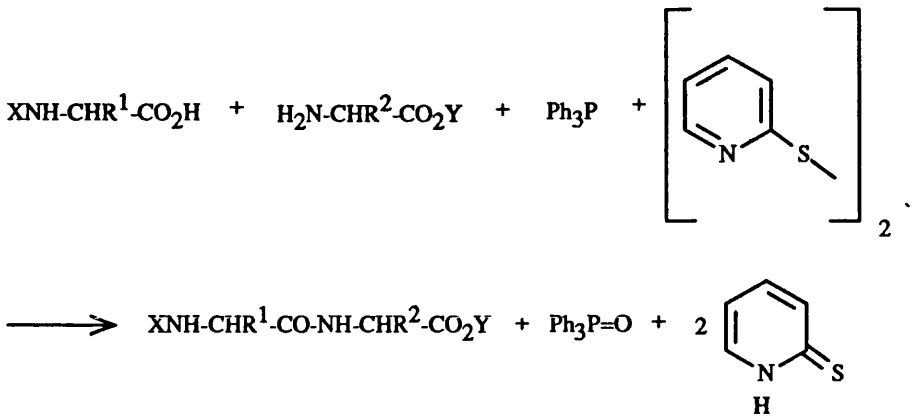


Dhbt ester

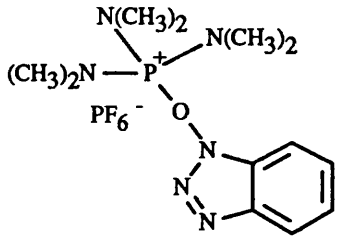
(3)

Other suitable coupling reagents include diphenylphosphoryl azide (DPPA)[40a] and diethyl phosphorocyanidate (DEPC).[40b] In addition, the oxidation-reduction condensation method developed by Mukaiyama[41] is applicable to standard stepwise solid phase synthesis, where a combination of triphenylphosphine and 2,2′-dipyridyl disulphide is employed, as shown in **Scheme 3.**

Further success has been achieved by application of the benzotriazolyloxy*tris*(dimethylamino)phosphonium hexafluorophosphate (BOP) reagent[42] (4).



Scheme 3



(4)

1.1.7. Monitoring

A successful solid phase synthesis relies on efficient and reliable monitoring procedures to ensure that coupling reactions have proceeded to completion.

Widely adopted approaches are the highly sensitive ninhydrin[43] and 2,4,6-trinitrobenzenesulphonic acid (TNBSA)[44] tests which indicate the presence of unreacted free amine by a colouration response. A highly accurate variation of the ninhydrin test has also been developed.[45]

A system for the continuous-flow spectrophotometric monitoring of the Fmoc-polyamide solid phase method has been developed,[46] allowing acylation and Fmoc deprotection reactions to be followed by observing absorption of the fluorenyl derivatives. In addition, by application of Dhbt esters, complete acylation reactions are indicated by fading of the transient yellow colour initially produced by reaction of the liberated hydroxy component with unreacted, resin bound amine.

Applications of gel phase ^{13}C and ^{19}F n.m.r. have been reported. However, such processes are both time consuming and inherently lacking in sensitivity.

Another useful procedure employs the total hydrolysis of aliquots of peptidyl-resin removed after the synthetic steps. In this regard, the technique of using internal reference amino acids is often very useful for the accurate determination of yields and retention of chains on the support.

1.1.8. Cleavage and Deprotection

Upon completion of the assembly, the peptide must be cleaved from the resin. During this step, side-chain protection may also be removed depending on the cleavage reagent used.

The common acidic cleavage reagents include HF, trifluoromethanesulphonic acid (TFMSA)-thioanisole and TFA. Cleavage under effectively neutral conditions may be effected by thiols or photolysis (350 nm), as appropriate to the linkage in question. In addition, the use of different nucleophiles enables a range of C-terminal functionalised peptides to be obtained. Thus, aqueous or alcoholic hydroxide yields the peptide acid; alcohols, in the presence of tertiary amines, affords the peptide ester; amines, the peptide amides; and hydrazine, the peptide hydrazide. It is also possible to use α-amino acid esters to cleave some of these linkages, thus adding an extra residue at the C-terminus, or to employ the N^{α}-amino group from the same molecule for intra-molecular cyclisations.

Acid cleavages are always carried out in the presence of a carbonium ion scavenger such as anisole, to prevent alkylation side reactions. For more resistant anchoring bonds and protecting groups, a combined "low-high" HF procedure has been developed to provide better product purity.[47] Thus, using a modified cleavage mixture of HF-dimethyl sulphide-*p*-cresol (low HF concentration) most of the precursors of harmful carbonium ions are removed before the final strong acid step employing HF alone. Recently, trimethylsilyl trifluoromethanesulphonate (TMSOTf)-thioanisole[48] has been found to be an extremely effective reagent for the cleavage of a peptidyl-resin linkage and a number of protecting groups.

Side reactions in peptide synthesis often occur as a consequence of the cleavage (as explained in a subsequent section). Occasionally, it is advantageous to design the synthesis such that cleavage from the resin and removal of all or some of the side-chain protecting groups occurs in separate steps.

1.1.9. Purification and Characterisation

High performance liquid chromatography, particularly in the reverse phase (RPHPLC), has proved to be extremely valuable for the purification of peptides, enabling high resolution and effective discrimination of impurities. Other useful techniques include ion-exchange chromatography, gel permeation chromatography, affinity techniques and counter current distribution.

Effective determination of product purity requires elucidation of homogeneity in several chromatographic systems. Correct amino acid composition upon hydrolysis, consistent with spectroscopic properties, is required. Proton n.m.r. is also useful if the peptide is small enough and available in sufficient quantities. However, a powerful technique is fast atom bombardment (F.A.B.) mass spectrometry, which enables the visualisation of molecular ions in excess of 5000 a.m.u., and the determination of other sequence information.

1.1.10. Problems and Side Reactions

Problems may arise not only during the solid phase synthesis, but also as a consequence of the final cleavage.

At every deprotection/coupling cycle, any errors have a cumulative effect, thus it is imperative that each must proceed near-quantitatively. Impurities in any amino acid derivatives or in other reagents or solvents, which may react with resin bound product, must therefore be removed. It has been suggested that incomplete deprotection and coupling reactions may be due to inter- or intra-chain interactions. It has recently been found that inclusion in the coupling mixture or pre-treatment of the peptidyl-resin with solutions of salts known to disrupt peptide structure, gives major improvements in coupling efficiency in certain difficult sequences.[49] If

coupling reactions remain incomplete, then chain termination must be effected by acetylation. In the case of incomplete deprotections, chain growth can later resume and deletion peptides arise, lacking the intervening residue. Reversible reactions between the terminal free amine and functional groups on the resin may also be problematic. Growing chains may also be irreversibly blocked resulting in formation of terminated peptides. In addition, premature loss of chains from the resin or late initiation of chains onto superfluous functional sites, are further sources of cumulative errors.

Another potential cause for concern is racemisation. Although this does not appear to occur during normal stepwise synthesis, the activation of a C^{α}-carboxyl group, or esterification of an N^{α}-urethane protected amino acid to benzyl alcohol handles or supports, has been reported to result in as much as 10% racemisation in some cases.[27] The reaction conditions must therefore be carefully controlled.

The introduction of certain amino acids may also cause problems associated with irreversible modifications or rearrangements.[50] For example, attempted incorporation of the third amino acid of a sequence may result in diketopiperazine formation, since the free N^{α}-amino group of the anchored dipeptide can attack the C-terminal carbonyl. Certain residues, in particular glycine, are particularly susceptible but generally the problem may be controlled. During cleavage, strong acids such as HF and TFMSA can promote succinimide formation on removal of benzyl ester protecting groups of aspartic acid. Opening of the ring may occur in either direction, to give the desired peptide and a side product with the peptide chain extending from the β-carboxyl. The N → O acyl shift at serine and threonine is also affected by this acidic treatment, though the process may be reversed by pH control. Further problems include intramolecular C-alkylation at tyrosine, involving migration of the phenolic protecting group, oxidative degradation of tryptophan and tyrosine, and oxidation and alkylation of methionine and cysteine.

1.1.11. Synthetic Achievements

Since the synthesis of the first biologically active peptide, bradykinin,[51] there has been a multitude of syntheses of naturally occurring peptides and their analogues. In **Table 1**, a few examples are given to illustrate the range of biological problems that can be approached by solid phase synthesis. Techniques are outlined which may be applied for the formation of disulphide bridges, other cyclic systems, and peptides containing unnatural residues. It is evident that forty or fewer residues, as well as longer sequences, can be reliably assembled. A most interesting and recent development is the solid phase synthesis of glycopeptide sequences,[52] required for elucidating the functions of these biologically important compounds.

1.1.12. Conclusions

In summary, since Merrifield's initial report on solid phase peptide synthesis, the field has developed immensely. This is reflected by new, milder, orthogonal protection schemes and efficient coupling methods. Chemical problems associated with the repetitive stepwise procedure have been largely solved by improvements in resins, functionalising procedures, anchoring linkages and reaction protocols. Development of more efficient cleavage reagents and peptide purification techniques have also been significant.

Although automated systems exist, the success of any peptide synthesis is governed by the validity of the underlying chemistry. Developing areas include use of solid supports for segment condensations, and anchoring of amino acids through their side chains to facilitate both mono- and bi-directional chain extension. In addition, the simultaneous application of both liquid phase and solid phase supports for dual anchoring, and the effects of the polymer on solid supported chemical reactions are also of interest.

Despite recent advances in molecular biology, solid phase synthesis is often the method of choice for the production of peptides. Though the underlying mechanisms responsible for the extraordinarily high biological potencies of certain peptides remains poorly understood, many attempts have been made to determine the essential structural and conformational

Table 1

Some Peptide and Protein Like Molecules Synthesised by the Solid Phase Method.

Compound	Number of Amino Acids	Remarks
conformationally restricted enkephalin analogue	5	side chain to side chain cyclisation performed on resin-bound peptide; extra level of selectivity reached in protection scheme
oxytocin, vasopressin	9	ammonolysis of standard benzyl ester-resin or acidolysis of MBHA resins; disulphide formed by oxidation of dilute solutions
deamino-oxytocin carba-analogues	8	20 membered ring; formed on resin-bound peptide; cleavage by ammonolysis
adrenocorticotropin (ACTH)	39	fully active hormone in 3% isolated purified yield, made via Boc-polystyrene methodology
parathyroid hormone	84	made with PAM resin, 12% purified yield
β-lipotropin	89	prepared both by stepwise assembly, and by condensation in aqueous solution or protected segments made on the solid support
ribonuclease A	124	first synthesis of material with nearly full enzymatic activity in 3·5% isolated purified yield

features that are required for receptor binding and activity.

1.2. DETERMINATION OF STRUCTURE-CONFORMATION-ACTIVITY RELATIONSHIPS

The interaction of a peptide hormone with specific macromolecules is thought to cause activation leading to a triggering of physiological activity.

It is clear that the mechanism of peptide hormone action differs from that of enzymes; i.e. such hormones do not generally possess an active site comprising a few vital amino acid residues. Instead, several discrete sequences of adjacent amino acids seem to be responsible for differing components of the total biological activity spectrum. Information for biological activity may also reside in separate residues of the sequence, which are brought into close proximity by conformational adaptation. In the cases of insulin and the oxytoxin-vasopressin group of hormones, the major function responsible for their biological specificities and potencies appears to be the overall conformation.

Efforts to understand the precise structural, conformational and dynamic properties of peptides which elicit multiple biological effects are at the forefront of modern approaches to develop a rational approach to drug design. For this purpose, various methodologies have been applied.

1.2.1. Classical Structure-Activity Studies

Relationships between peptide structure and biological activity can be readily determined by performing structure-activity studies, involving deletions and/or substitutions of amino acids comprising the primary sequence of the peptide. Replacements may be designed to alter particular functional groups and any change in potency may indicate the importance of the substituted amino acid for activity.

Although these are important preliminary analyses, structure-activity studies alone rarely provide insights into the bioactive conformation.

1.2.2. Conformational Studies of Bioactive Molecules

Primary conformational analysis of the native peptide hormone and

closely related analogues, involves the application of various techniques. These have included X-ray crystal structure analyses, nuclear magnetic resonance, circular dichroism, infrared spectroscopy and theoretical calculations. Such methods have given an insight into the accessible conformations and permitted some elaboration of novel rationales for analogue design.

Efforts to relate conformation to biological activity have been difficult due to a number of inherent limitations. In particular, since most of the peptide hormones and neurotransmitters are small linear peptides, they have considerable conformational freedom. Cyclic peptides containing biologically active sequences are more suitable models where the usual flexibility of the peptide chains is reduced. The existence of several different conformations has to be taken into account. Conformational rigidity will be restricted to certain portions of the molecule and mobility inherent in other parts. The extent of conformational homogeneity must be established and of all the spectroscopic methods available, high resolution n.m.r. has emerged as a particularly powerful technique.

1.2.3. N.M.R. as a Method for Conformational Analysis of Peptides

The conformation of the peptide backbone can be expressed in terms of the torsion angles ϕ,φ and ω, while the side chain can be characterised by the angle χ **(Figure 8)**.

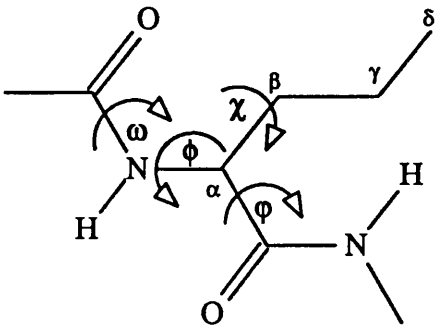


Figure 8

For the purpose of assigning signals to amino acids, the search for coupling patterns has been enormously simplified by the use of modern 2-D n.m.r. techniques[53], such as ^{1}H-^{1}H and ^{1}H-^{13}C Correlation Spectroscopy (COSY). It is possible to obtain sequence information by means of Nuclear Overhauser Effects (NOE) through NOE-difference, 2D NOE (NOESY) and Rotating-Frame NOESY (ROESY) experiments.[53,54] Some of the available techniques for determining conformation are summarised in **Table 2.**

First indications concerning molecular structure are provided by a study of the NH shift as a function of solvent and temperature. The orientation of amide linkages is generally defined by hydrogen bonding (intra- or inter-molecular, or by interaction with the solvent). Intermolecular hydrogen bonds, and those to the solvent, are readily cleaved with increasing temperature. The most meaningful results have been obtained in DMSO solution. In this solvent, temperature gradients in excess of 3ppb/°C are considered evidence of external NH orientation, whereas those under 2ppb/°C point to solvent shielding.

In using ^{1}H n.m.r. spectroscopic chemical shifts, anisotropic effects from neighbouring carboxyl groups or aromatic side chains may be identified as a source of information on molecular geometry. Thus the orientation of an α-proton of one amino acid residue in the plane of the carboxyl group of the adjacent amino acid leads to a considerable shift to lower field.

Homonuclear intramolecular NOEs between protons are caused by a dipolar relaxation mechanism, and there is a correlation between the enhancement observed and the distance between these protons. In one dimensional n.m.r., these effects manifest themselves by changes in signal intensity following irradiation at the resonance frequency of the interacting nucleus. NOEs can best be detected by application of the modern 2D techniques of NOESY and ROESY.

Of interest are ^{3}J (NH-$C^{\alpha}H$) coupling constants, which provide constraints on the backbone dihedral angle ϕ. A 2D n.m.r. method has been developed for their accurate measurement.[55] A recent report also relates the C^{α}-proton chemical shifts to secondary structure in peptides.[56]

Table 2

Possible Methods of Determining the Conformation of Peptides by N.M.R. Spectroscopy

Cause	Observed Spectral Parameter		Typical Information
interaction with environment	N-H shift as a function of solvent, temperature		orientation of the amide bond, H bonds
interactions through bonds	scalar coupling :	vicinal coupling	: angles ϕ,φ,χ
	:	geminal coupling of glycine $C^{\alpha}H$	: angle χ
	:	direct coupling ^{13}C-H	: C^{α}-CO angle
	:	direct coupling ^{15}N-H	cis/trans -isomers of the amide bond
interactions through space	chemical shift :	^{1}H (anisotropic)	orientation of the aromatic side chains
	:	^{13}C (steric and electronic effects)	cis-trans-isomerisation at proline
	dipolar coupling :	NOE effect (^{1}H)	intra-molecular proton distances
mobility	line coalescence		processes with relatively high barriers
chemical exchange	H/D exchange rates		extent of exposure of NH groups

1.2.4. Conformational Homogeneity in Solution

In general, differences in chemical shifts, coupling constants, and temperature dependence between different amino acids in the peptide sequence can be used as indications of conformational homogeneity. The smaller these differences, the greater the possibility that a rapidly established conformational equilibrium will cause an averaging of the parameters. The main indications for conformational homogeneity are summarised as follows:

(i) strong differentiation of temperature gradients of the NH signals between separate amino acids units,
(ii) strong differentiation between chemical shifts of like amino acid units,
(iii) large chemical shift differences between geminal diastereotopic glycine protons,
(iv) strong differentiation between vicinal coupling constants of diastereotopic protons,
(v) large differences between NH-$C^{\alpha}H$ coupling constants.

Conformational information obtained may be supplemented by additional means, whether experimental or theoretical. In the latter case, molecular dynamics is a particularly useful technique.

1.2.5. Molecular Dynamics and Conformational Energetics

Theoretical methodology, for use in conjunction with experimental procedures, may be applied for elucidation of accessible conformations and associated flexibility, conformational transitions and dynamics. Molecular dynamics and energy minimisation techniques[57] make possible a description of the conformational properties of the peptide in terms of the precise position of atoms, their fluctuations in time, and the inter-atomic forces acting on them. Possible active conformations may be identified which can be compared to those suggested by independent n.m.r. studies. Experimental distances obtained from the n.m.r. experiments may also be introduced as NOE constraints, to refine the accessible conformations.

The combination of n.m.r. and molecular modelling may indicate particular substitutions that can be performed which may result in further conformational restrictions.

1.2.6. Conformational Restrictions of Biologically Active Peptides

It is necessary to decipher whether the conformations determined in solution have any relevance to the conformation in the biologically active state. Thus, it is beneficial to minimise such problems by use of conformationally constrained analogues.

Conformational restrictions may be introduced[58] by local constraints, imposed by sterically constrained amino acids. For this purpose, α,β-dehydro-, N-methyl-, cyclopropyl-, α,α-dialkyl- and β,β-dialkyl-amino acids have been used with considerable success.[59] In addition, bicyclic and ring contracted analogues may also be formed.

Constraints can also be introduced by incorporation of moieties designed to mimic certain conformational features. Recently, a novel spirocyclic unit was introduced, by solid phase methodology, as a β-turn constraint.[60]

Thus, these conformationally constrained analogues may be studied by a combination of bioassay, n.m.r. spectroscopy, and molecular dynamics to identify the important conformational features of the parent peptide.

1.2.7. Conclusion

In summary, n.m.r. spectroscopy is an excellent probe for recognising molecular conformations, due to its sensitivity to individual atoms in their specific environments. This has been aided by considerable advances in 2D techniques.

We envisaged that some of the techniques detailed above may be applied in the structure-conformation-activity study of the melanin concentrating hormone.

1.3. MELANIN CONCENTRATING HORMONE (MCH)

Many lower vertebrates undergo a change of body colour in response to background conditions through dispersion and concentration of melanin granules (melanosomes) within integumentary melanophores. It has been

postulated that these colour changes are exhibited through dual hormone control by two mutually antagonistic pituitary melanophorotropic hormones.[61]

It is well known that pigment dispersion, giving rise to skin darkening, is regulated through α and β-melanin stimulating hormone (MSH). Much is known about the biosynthesis, structure-activity and control of those melanin dispersing hormones, which are derived from the parent pro-opiomelanocortin (POMC) molecule, together with the opioid peptide, β-endorphin, and other related peptides.

The hormone antagonistic in function to MSH has proved more elusive. It was first substantially proven to exist in the pituitary and hypothalamus of teleost fish,[6] though evidence for existence in amphibia was not originally forthcoming. Since the hormone causes a blanching of skin colour by effecting aggregation (concentration) of pigmentary organelles it was, appropriately, named the melanin concentrating hormone (MCH). Melanosome aggregation in response to MCH is reversed by an equimolar concentration of α-MSH.

Recent work has confirmed the involvement of a hormone in colour change in fish,[2] and shown that it is a neuropeptide synthesised by neurones in the hypothalamus.[7] In the case of fish, it passes in axons to the posterior pituitary, where it is released into the circulation to achieve its effect.

The peptide causes melanosome concentration only in fish. In the frog (*Rana pipiens*) and the lizard (*Anolis carolinensis*), MCH has the opposite effect and causes dispersion of melanosomes,[3] an action suggested to be mediated through MSH receptors. Thus MCH exhibits both melanosome concentrating and dispersing actions, depending on the species studied.

MCH bioactivity and immunoreactivity has also been found in the brains of higher vertebrates, where the peptide apparently exerts physiological effects unrelated to colour change. Thus, it may serve as a regulator of pituitary function; in fish it is found to depress release of ACTH from the pituitary both *in vivo* and *in vitro*; and in rats, it exerts this effect *in vitro*, but only at higher concentration.[4] MCH has been found to stimulate the release of growth hormone,[5] and immunocytochemical studies on fish, amphibia and mammals, show that numerous axons protrude from

the hypothalamic perikarya into other regions of the brain, where the hormone presumably exerts a neuromodulatory effect.

Teleost MCH (**1**) was characterised [7a] on isolation in pure form from salmon pituitaries, and was effective at nanomolar concentrations in stimulating melanosome aggregation in teleost fish.

(1)

The structures of two kinds of mRNA encoding the salmon MCH have been elucidated.[62] The MCH heptadecapeptide was present at the carboxyl terminus of a putative MCH precursor consisting of over one hundred amino acid residues.

1.3.1. Reported Syntheses of MCH

Salmon MCH has been synthesised by both conventional solution and solid phase methodologies.

The solution phase synthesis[9] proceeded via successive condensation of four peptide fragments and the N-terminal amino acid. Following cleavage of protecting groups with TFMSA-thioanisole, disulphide bond formation was effected by air oxidation.

The first solid phase synthesis of MCH was performed by Wilkes and co-workers,[10] by application of the Boc-polystyrene methodology. Cleavage of the peptide from the resin and removal of all side-chain protecting groups was accomplished by treatment with HF. Treatment of the crude disulphydryl peptide with potassium ferricyanide[63] resulted in cystine bond formation to yield the purified peptide in 14% overall yield.

MCH has also been synthesised by the continuous-flow Fmoc-polyamide solid phase method.[11] The cysteine residues at positions 5 and 14 were protected as the acetamidomethyl (Acm) derivatives. Treatment of the complete peptidyl-resin with TFA furnished the *bis*-acetamidomethyl heptadecapeptide. Disulphide bond formation was elicited simultaneously with Acm cleavage by treatment with iodine.[64]

The synthetic products stimulated melanosome aggregation in teleost fish in concentrations comparable to the natural hormone.

1.3.2. Comparisons of Structure-Activity Studies

The bioactivities of various MCH fragments have been assessed using either synthetic sequences or products of enzymatic cleavage of the natural hormone. Collective results from studies by various workers are shown in **Table 3.**

Chemical and enzymatic modifications of MCH were conducted by Kawazoe et al[8] to deduce the essential amino acid residues for bioactivity. When the N-terminal residues were removed by Edman degradation, the resulting fragment MCH(5-17) (**5**) was found to have full activity. Carboxypeptidase digestion of MCH gave MCH(1-14) (**6**) which also showed full bioactivity. Indeed, complete potency was also retained in the MCH(5-14) (**7**) fragment. Modification of Trp^{15} by *o*-nitrophenylsulphenyl chloride to yield [NPS-Trp^{15}]MCH did not alter the potency. Modification of Tyr^{11} by tetranitromethane to form [NO_2-Tyr^{11}]MCH caused significant reduction of activity. Oxidation of the $Met^{3,6}$ residues with hydrogen peroxide to afford [O-$Met^{3,6}$]MCH caused partial loss of the activity. However, reduction and carboxamidomethylation to form [Acm-$Cys^{5,14}$]MCH resulted in a complete loss of potency. The results suggest that the disulphide bridge is essential for activity and that Tyr^{11} may be located in the active region.

In studies carried out by Baker et al,[12] the derivative [O-$Met^{3,6}$]MCH possessed virtually full bioactivity, greater than that detected by Kawazoe et al. The iodinated Tyr^{11} analogue [I-Tyr^{11}]MCH had only marginal activity, further reflecting the importance of the Tyr^{11} residue. Replacement of Met^3 and Met^6 by norvaline (NVa) or propargylglycine (Pra) also resulted in reduction of activity, but not drastically so. The two

Table 3

Structure Activity Studies of Salmonid MCH+

Peptide	Baker et al(a)	Kawazoe et al(b)	Hadley et al(c)
MCH	100	100	100
cyclic MCH(5-17)	-	100	100
cyclic MCH(1-14)	-	100	10
cyclic MCH(5-14)	-	100	1·0
[Acm-$Cys^{5,14}$] linear MCH	0·3	0·0	-
[O-$Met^{3,6}$]MCH	88	10	-
[NPS-Trp^{15}]MCH	-	100	-
[$NVa^{3,6}$]MCH	37	-	-
[$Pra^{3,6}$]MCH	15	-	-
[NO_2-Tyr^{11}]MCH	-	0·1	-
[I-Tyr^{11}]MCH	0·25	-	-

+ values are the % potencies compared with the native hormone which is taken as 100%

(a) tested on scales from *Ctenopharynogodon idellus*

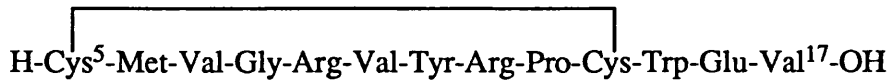
(b) tested on scales from *Sarotherodon mossambicus*

(c) tested on skin from *Synbranchus marmoratus*

Acm = acetamidomethyl; NPS = *o*-nitrophenylsulphenyl; Pra = proparglyglycine; NVa = norvaline

methionine residues may, therefore, not be crucial for biological activity. In contrast to the findings of Kawazoe,[8] the [Acm-Cys^{5,14}] linear MCH was a full MCH agonist, though with a potency of only 0.3%.

Fragment analogues of the native peptide have also been studied by Hadley et al[13] in an attempt to decipher the differential structural requirements for the MCH- and MSH-like activities. In the fish-skin assay, MCH(5-17) (**5**) was equipotent to MCH but MCH(1-14) (**6**) and MCH(5-14) (**7**) possessed 10% and 1% activities respectively, this in contrast to the findings of Kawazoe.



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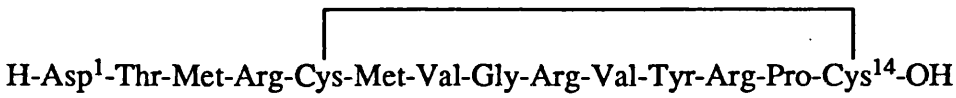

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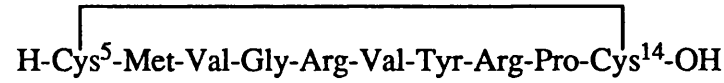


(7)

In the MSH frog and lizard skin bioassay, where the abilities of analogues to effect melanosome dispersion is studied, the 5-17 and 5-14 fragments of MCH were inactive, whereas the 1-14 sequence exhibited minimal MSH activity. MCH is self-antagonised in a dose-response manner by higher concentrations of the peptide, as was the activity of MCH(1-14) (**6**). The results indicate that the N-terminal sequence is particularly critical to MSH-like activity, whereas MCH activity is more a function of the C-terminal exocyclic residues.

Lebl has studied the MCH-like agonist and antagonist activities of ring

contracted analogues.[14] The disulphide cyclised portion was contracted from the 5-14, to the 7-14, 8-14 and 10-14 residues with concomitant substitution of alanine for cysteine at position 5. Similar substitutions were also made in the 5-17 analogue. Contraction of the ring generally resulted in loss of MCH-like activity, further reflecting the importance of the disulphide bond. However, some analogues still possessed MSH-like activity. These, like α-MSH, also antagonised the actions of MCH on fish melanosomes. In common with α-MSH, the antagonist activity of these analogues were Ca^{2+} dependent. The evidence suggests that the antagonism of MCH activity is related to the intrinsic MSH-like activity of the analogue. This indicates that MCH and α-MSH may be structurally and evolutionary related. Indeed, sequence homology between the α-MSH(4-10) fragment (**8**) and MCH (**1**) is evident, as shown in **Figure 9**, and is supported by the demonstration that immunoreactive MCH and α-MSH may co-exist in the same neurosecretory granules within the fish and hypothalamus of the rat.[65]

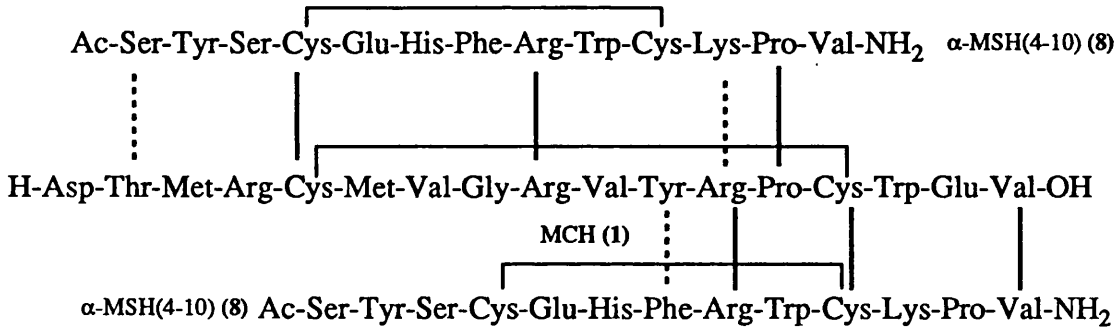


Figure 9

The nature and physiological significance of the MCH-like substance in the mammalian brain is as yet unreported, though it is currently being purified and characterised.[15a] The low bioactivity of the mammalian hormone when tested in the fish melanophore bioassay, and relatively poor immunoreactivity with antiserum raised against the fish MCH, suggest that the mammalian hormone is structurally different.

The sequence of rat MCH (9) has recently been determined[15a] and shows a close homology to salmonid MCH (1) (**Figure 10**)

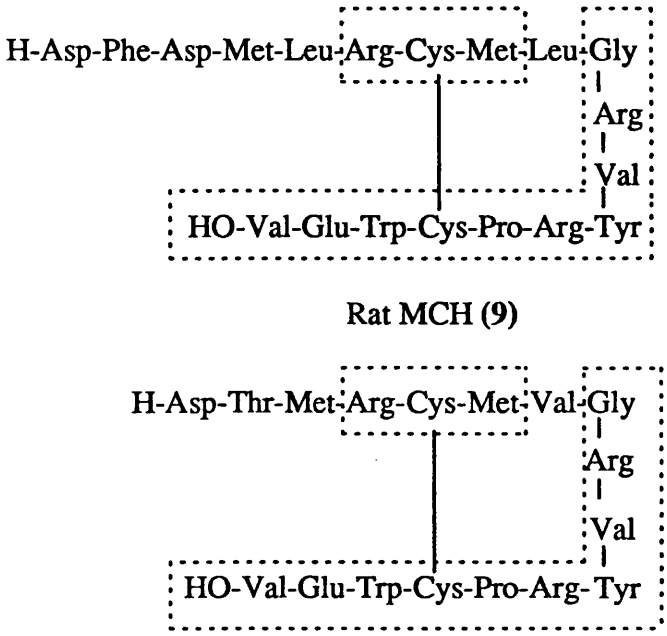


Figure 10

Like other brain neuropeptides, the ubiquitous distribution of cell bodies and fibres showing presence of MCH, indicates a modulating action on a variety of functions. Indeed, recent developments have suggested that peptides of this size possess more structural integrity than was previously thought.[54,66]

1.4. AIMS

It is suggested in the previous discussions, that MCH is a potent pituitary regulating hormone. Thus, it is highly desirable to learn more about its methods of action since potential applications may involve the definition of human receptor sites related to those of fish. The control of ACTH and growth hormone may also be in prospect.

Some of the techniques already discussed have been applied in a structural and conformation study of MCH, where the syntheses of rationally designed analogues were performed by the solid phase methodology.

An initial structure-activity study was required to determine the essential structural elements. It is possible to predict accessible conformations independently by high field n.m.r. and molecular dynamics studies of selected analogues. The results from the theoretical study can then be used in conjunction with experimental findings to determine the conformational features necessary for binding and activity. These might be further probed by the analysis of molecules of restricted conformational freedom.

Therefore, such a structure-conformation-activity study may lead to some understanding of the receptor site requirements. Indeed the ultimate aim is the design of an effective non-peptide agonist or antagonist for MCH, since this may prove to be extremely useful in a variety of pharmacological and physiological applications.

RESULTS AND DISCUSSION

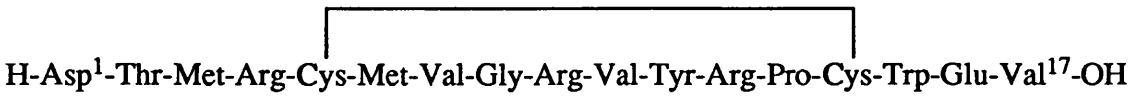
RESULTS AND DISCUSSION

Chapter 2

SYNTHESIS, BIOLOGICAL ACTIVITY AND CONFORMATIONAL STUDIES

"STAGE 1"

The synthesis of each analogue of MCH (**1**), required for the preliminary structure-conformation-activity study, is considered in the following sections. The rationale for each preparation is also discussed.



(1)

2.1. GENERAL SYNTHETIC PROCEDURES

Before considering each synthesis in detail, it is necessary to review the common methodologies employed.

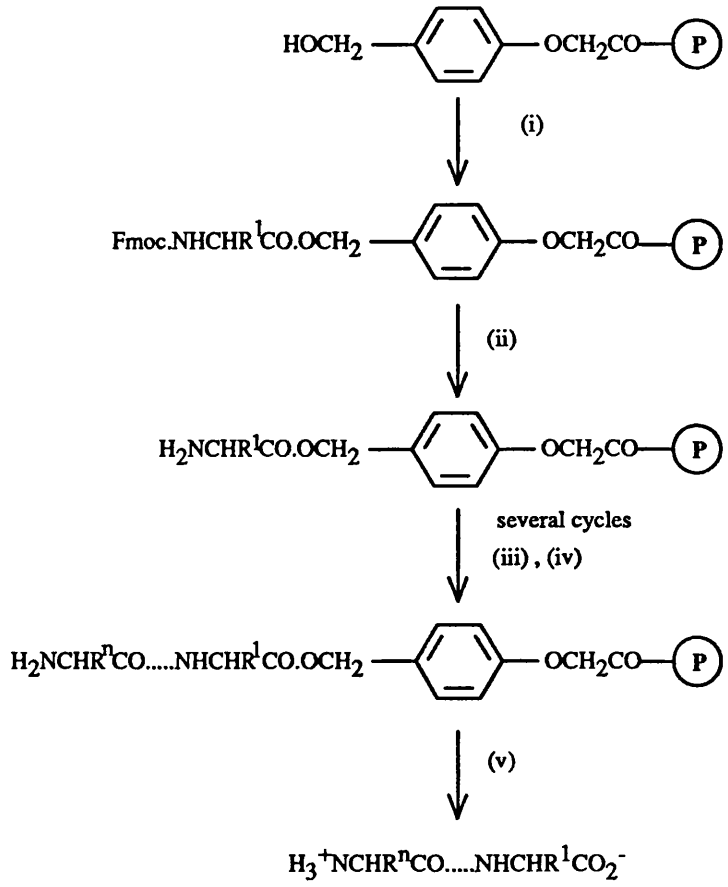
2.1.1. Solid Phase Strategy and Tactics

All solid phase assemblies were performed using the continuous-flow Fmoc-polyamide method.[24]

Both the conventional Boc-polystyrene and Fmoc-polyamide procedures have been applied in previous syntheses of MCH.[10,11] We opted for the latter since we considered certain intrinsic features of this method to be advantageous.

Use of the base labile N^{α}-Fmoc protecting group in combination with both acid labile side-chain protection and peptide-resin linkage agent, enables the solid phase synthesis to be carried out under milder conditions than in the Boc-polystyrene procedure. This chemical selectivity further widens the scope of the method. The use of the polyamide gel support allows the polar aprotic solvent, DMF, to freely permeate the medium, therefore providing an environment within the polymer matrix which is

substantially optimised for the chemical processes involved. It has been reported[30] that a peptide was successfully prepared by employing DMF, where synthesis had failed using the less polar medium, DCM. The composite polydimethylacrylamide-macroporous kieselguhr support permits low pressure, continuous-flow operation. This is advantageous since removal of excess reactants is inherently more efficient than with batchwise washing. Perhaps more importantly, flow systems offer a greater opportunity for analytical control. Continuous spectrophotometric effluent monitoring is easily achieved through the high u.v. absorption of certain chromophores eg. that of Fmoc derivatives. A summary of the general strategy adopted is outlined in **Scheme 4.**



(P) = -Nle-NHCH$_2$CH$_2$NH-CO-polydimethylacrylamide

(i) Fmoc-amino acid : anhydride / Pfp ester , Dmap (ii) 20% piperidine-DMF

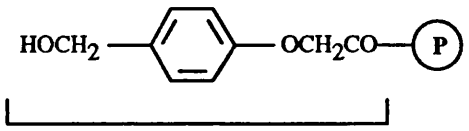
(iii) Fmoc-amino acid : Pfp ester , HOBt / anhydride / Dhbt ester

(iv) 20% piperidine-DMF (v) TFA , scavengers.

Scheme 4

2.1.1.1. Resin Support

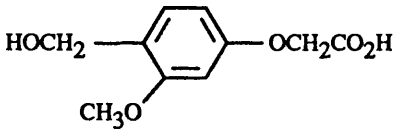
Each synthesis was performed using the composite resin allowing continuous-flow operation. In almost every case we employed the 4-hydroxymethylphenoxyacetyl-norleucyl-derivatised polydimethylacrylamide-kieselguhr support **(10)**. The handle provides lability to 95% TFA to afford the C-terminal carboxylic acid of the peptide and to effect removal of the majority of side-chain protecting groups. In the main, peptides were required in this form. Alternative handles, 4-hydroxymethyl-3-methoxyphenoxyacetic acid **(11)** and 4-hydroxymethylbenzoic acid **(12)**, provide lability to 1% TFA and amines respectively.



Handle

P = -Nle-NHCH$_2$CH$_2$NH-CO-polydimethylacrylamide

(10)



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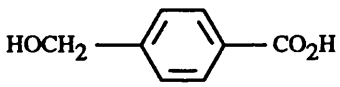


(12)

2.1.1.2. First Residue Attachment

The first residue was incorporated using a 4-fold excess of the preformed symmetrical anhydride, or Pfp ester, of the Fmoc-amino acid in the presence of DMAP as the effective catalyst.

All Fmoc-amino acid anhydrides were prepared in the traditional manner from the corresponding Fmoc-amino acid and DCC in DCM solution. In general, filtration and evaporation afforded the crystalline anhydride directly, contaminated only with traces of DCU. Some

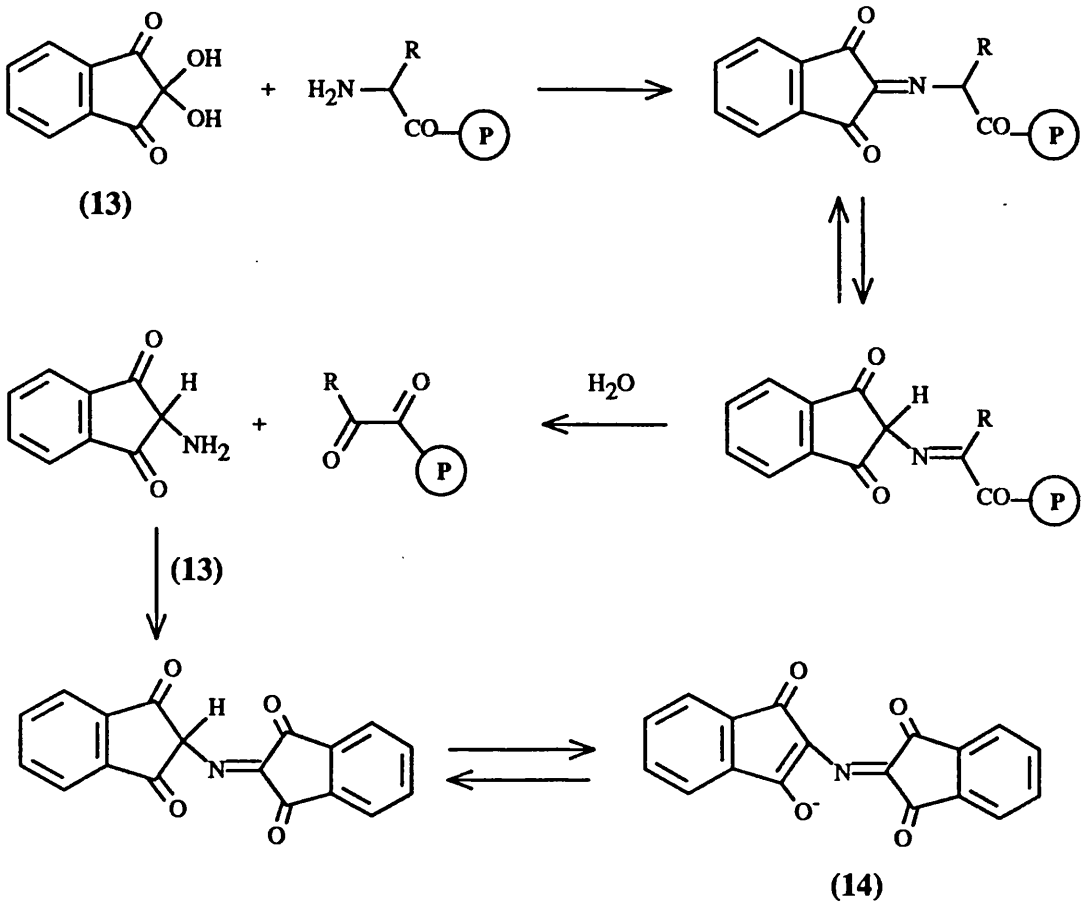
anhydrides were observed to precipitate during their formation, but the majority remained in solution. Slightly less than the theoretical amount of DCC required was employed in order to ensure that no excess should be present as a contaminant in the isolated anhydride, since this may encourage side-reactions or induce base-catalysed decomposition of the anhydride. Each derivative was prepared immediately before use.

Conditions have been established for minimising DMAP-promoted racemisation during introduction of the first Fmoc-amino acid.[27] To this end, it was common practise to add DMAP, as a dilute solution in DMF, to the resin after the Fmoc-amino acid derivative. This procedure ensured that the two reactants were mixed in the presence of the hydroxy component and not beforehand. The converse order of addition has been found to be less effective since DMAP had the initial tendency to remain at the top of the resin bed. The amount of DMAP used was also minimised and in general 0.1 molar equivalents of the amino acid derivative were satisfactory.

The method of activation used for the first-residue coupling was found to depend on the Fmoc-amino acid involved and is considered in the following sections 2.2. and 2.3. In general, two consecutive batches of reactants were employed and each recirculated for a certain period. Further batches were added as required until satisfactory resin coverage had been attained. When incorporating the Fmoc-amino acid via Pfp ester activation, recirculation periods of 2 hours or longer were used, the time being extended for acylations that proceeded more slowly. For anhydrides, 50 minute periods were employed, since a longer duration was deemed to be ineffective as the anhydride is faster reacting yet somewhat less stable in the reaction medium.

2.1.1.3. Quantitative Ninhydrin Colour Test

In most cases the extent of resin esterification was monitored by the application of the quantitative ninhydrin colour test.[45] Since this is an assay for free amino groups, the Fmoc group of the coupled amino acid was first cleaved with 20% piperidine-DMF. Reaction of ninhydrin, **(13)**, with a primary amine is generally believed to result in generation of the chromophore Ruhemann's purple, **(14)**, **(Scheme 5)**, seen as a blue colour in solution.



Scheme 5

Under the conditions employed, maximum colour is developed within 5 minutes and remains stable for a further 10 minutes at 100°C. The chromophore has maximum absorbance at 410-570 nm, but the determination was carried out at 570 nm because this gave the lowest blank absorbance. For routine monitoring, an average effective extinction coefficient was recommended[45] and used to estimate the level of free amine.

There are, however, some inherent inaccuracies with this method. For an exact value of the percentage resin esterification it becomes necessary to establish the effective extinction coefficient, i.e. colour yield of the reaction, for the particular amino acid-resin (or peptidyl-resin) under study. Moreover, the formation of the chromophore is dependent on an equilibrium reaction (**Scheme 5**). The position of equilibrium determines the amount of chromophore detected in solution and thus inaccuracies may

be introduced by factors affecting this equilibrium process.

2.1.1.4. Quantitative Fmoc Test

In later syntheses, an alternative procedure to the quantitative ninhydrin assay was used, which may prove to be more accurate. The adduct formed between dibenzofulvene and piperidine on removal of the Fmoc group has a strong u.v. chromophore (λ_{max} = 275 nm, ϵ_{max} = 10,700). This property was used as the basis of the quantitative Fmoc test to determine the amount of protecting group liberated from a sample of resin-bound amino acid and hence the level of resin coverage achieved. A similar analysis has been carried out by Meienhofer et al.[67] From calibration studies based on Fmoc-Val, the number of nmol equivalents of Fmoc per cm^3 of 20% piperidine-DMF was correlated to absorbance units. The method was applied to a number of Fmoc-amino acid to resin coupling reactions and the results were comparable to those obtained by amino acid analysis and the quantitative ninhydrin assay. However, the Fmoc determination should be independent of the particular amino acid involved and hence more accurate than the ninhydrin test. In addition the protecting group is cleaved rapidly and quantitatively with no dependence on equilibrium processes.

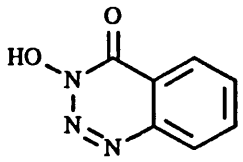
In both methods it is vital to wash the acylated resin thoroughly before application of the tests, since contaminants may give rise to misleading results.

2.1.1.5. Coupling of the Remaining Amino Acids of the Sequence

Once a satisfactory resin coverage has been achieved, the synthesis proceeds with the usual cycles of Fmoc cleavage and acylation; all Fmoc groups were removed by treatment with 20% piperidine-DMF. A 4-fold excess of the Fmoc-amino acid derivative was used in each case and was added in the minimum volume of DMF. In general, Pfp esters were employed, with added HOBt. Until recently, Fmoc-polyamide syntheses used symmetrical anhydrides[68] as the activated derivative for the majority of Fmoc-amino acids. Although very efficient, the use of anhydrides may be considered disadvantageous in that preparation is required for each derivative, immediately before use, since they can be somewhat unstable. The introduction of Pfp esters of Fmoc-amino acids to the polyamide-based

solid phase method[38] combines the high reaction rates and freedom from side reactions characteristic of Fmoc-amino acid anhydrides, with the crystallinity and stability on storage of active esters. Though it was our experience that Pfp esters with added HOBt are less reactive than symmetrical anhydrides, this was only problematic in certain cases, particularly attachment of the first Fmoc-amino acid to the resin.

The Pfp esters of Fmoc-Ser(*t*-Bu) and Fmoc-Thr(*t*-Bu) are exceptionally soluble and have not been obtained in a stable crystalline form. Early syntheses involving Fmoc-Thr(*t*-Bu) employed the anhydride. Dhbt esters now provide alternative activated derivatives,[39] and were subsequently employed for coupling of Fmoc-Thr(*t*-Bu), and also for Fmoc-Arg(Mtr), where the Pfp ester was not commercially available at that time. Reaction of Dhbt esters with resin bound amino groups results in the liberation of the hydroxy component **(15)**. A transient bright yellow colour



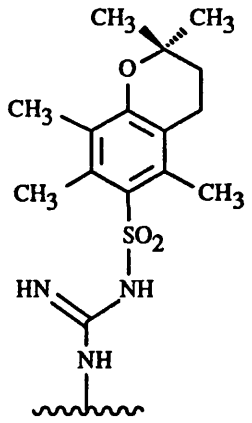
(15)

appears on the resin during the acylation which fades as the reaction proceeds. The yellow colour is attributed to ionisation of **(15)** by resin bound amino groups. Thus the Dhbt ester provides both an effective acylating agent and a sensitive indicator of the presence of unreacted amines. Reactivity parallels that of the Pfp/HOBt coupling.

2.1.1.6. Side-Chain Protecting Groups

The use of the Fmoc group enables the adoption of the acid labile *t*-Bu group for general amino acid side-chain protection. Thus, aspartic acid and glutamic acid were protected as *t*-Bu esters and threonine and tyrosine as *t*-Bu ethers. The intrinsically acid sensitive side chain of tryptophan was left unprotected. For cysteine, either the TFA-stable Acm[69] derivative or the TFA-labile trityl (Trt)[70] thioether were employed, depending on the permanence of S-protection required following cleavage of the peptidyl-resin. Use of the Acm derivative allowed purification of the linear

form. Both derivatives provided opportunity for disulphide bond formation simultaneously with protecting group cleavage. At the start of this study, a highly acid-labile protecting group for arginine was not available. Our search for a suitable candidate yielded only the N^{η}-(4-methoxy-2,3,6-trimethylbenzenesulphonyl) (Mtr) derivative,[71] though this required more vigorous conditions for the final cleavage (several hours contact with TFA) than were desirable, particularly when more than one arginine residue was present. Conflict with unprotected tryptophan was problematic. In most cases this was due to trapping of intermediates formed during the final deprotection. It has since come to our attention, that a more suitable candidate may be the N^{η}-(2,2,5,7,8-pentamethylchroman-6-sulphonyl) (Pmc) derivative[72a] **(16)**, as a TFA-labile protecting group for arginine with much enhanced lability over the Mtr group. We are yet to employ this derivative, however, it has proved successful in peptide syntheses elsewhere.[72] Presence of unprotected tryptophan could cause problems in an analogous fashion to Mtr deprotection, therefore care must be taken to have scavengers present.



(16)

2.1.1.7. Synthesis Monitoring

The progress of each synthesis was monitored continuously, at 330 nm, using a u.v. flow-cell in the reagent stream. An absorbance trace for a complete cycle of Fmoc-amino acid anhydride addition and deprotection is depicted in **Figure 11.**

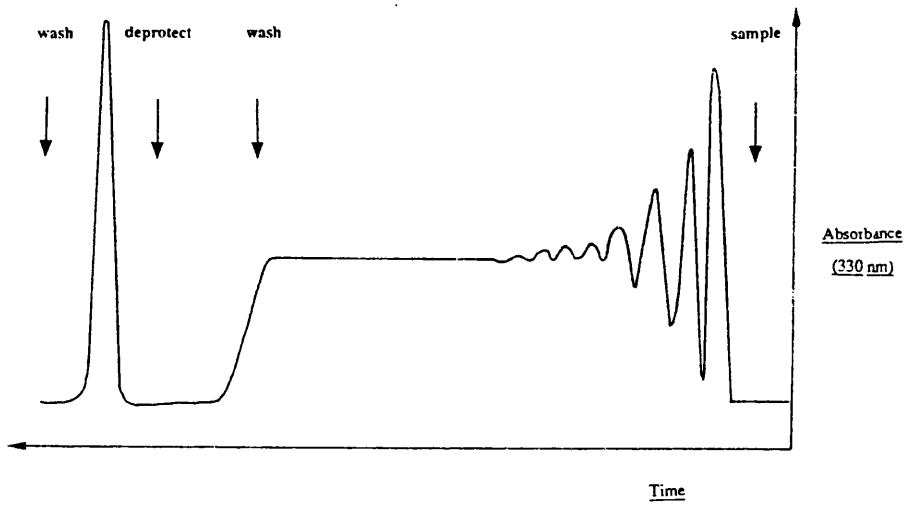


Figure 11

Introduction of the activated Fmoc-amino acid and recirculation through the resin produces the characteristic oscillating pattern. Progress of the acylation reaction can be followed by differences in the area of successive peaks as the Fmoc-amino acid anhydride recirculates through the resin bed and by the plateau level after a uniform concentration has been reached throughout the system. It is only possible to obtain useful quantitative information when using symmetrical anhydrides and in couplings other than the first-residue attachment, since the only major u.v. absorbing species are the Fmoc-amino acid anhydride and the free acid. The additional u.v. absorption of DMAP, aryl esters and in particular HOBt complicates, and reduces the value of, spectrophotometric monitoring. Quantitative monitoring of the deprotection reaction is still feasible. In normal operation, consistent areas of successive deprotection peaks provides assurance that the synthesis is proceeding smoothly. On certain occasions it was observed that the deprotection peaks were not consistent in shape. This occurred particularly during removal of the Fmoc group from proline, where a lower, broader peak was seen corresponding to a slower release of the chromophore into solution, most likely due to a reduced rate of cleavage of the Fmoc group in this case.

Routine monitoring of the acylation reaction was carried out by application of the qualitative ninhydrin colour test for residual free amine.[43] If an incomplete reaction was indicated, then recirculation of reactants was continued for a further period until a satisfactory test result was obtained. When using preformed symmetrical anhydrides however, a fresh batch of reactants was added and recirculated, since there was probably an insufficient amount of active anhydride remaining in the previous batch. This test is markedly less sensitive for the secondary amine group of proline and thus extended coupling periods were employed. When using Dhbt esters, the progress of the acylation reaction was further assessed by observing the resin. The removal of all excess reactants during the washing cycles was shown by the rapid fall in absorbance of the column effluent.

2.1.1.8. Apparatus

All solid phase peptide syntheses were performed on a semi-automatic microprocessor-controlled apparatus. The synthetic cycles are detailed in the Experimental section. The essential features of a continuous-flow synthesiser are shown in **Figure 12.**

The composite resin is placed in the reaction column(s), where the peptide assembly is carried out. The solvent/reagent to be passed through the resin is selected by a series of solanoid valves and delivered by a pump. Therefore, all chemical processes for Fmoc cleavage, washing, and addition of the activated Fmoc-amino acid derivative, are easily mechanised using the continuous-flow techniques. Use of the recirculation loop allows recycling of reactants through the reaction column, u.v. flow cell and valves 1 and 2 via the pump, so allowing the acylation reaction to proceed.

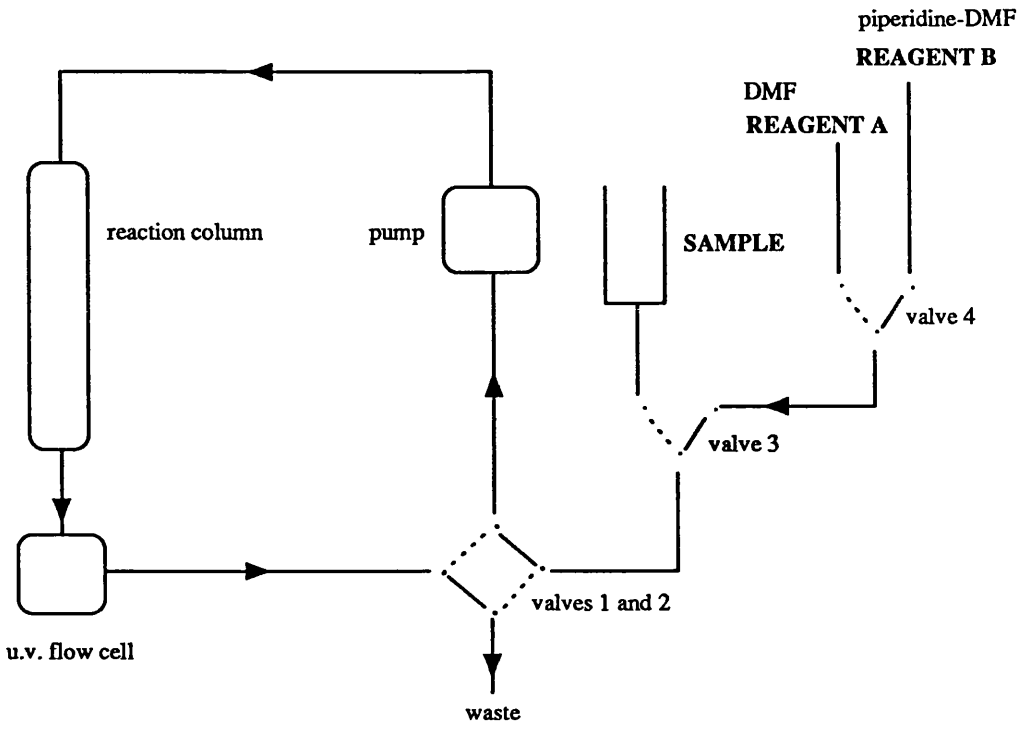


Figure 12

2.1.1.9. Cleavage/Deprotection

On completion of each assembly, the final Fmoc group was removed to afford the peptidyl-resin, which was then subjected to cleavage/deprotection. A preliminary small-scale cleavage was performed, monitored by analytical RPHPLC, to determine the optimum conditions e.g. choice of scavenging agents and reaction time. This is a prerequisite for successful syntheses.

A mixture of TFA, ethanedithiol (EDT) and phenol, was found to be satisfactory for most peptidyl-resins in this study. The TFA effected efficient cleavage of peptide from the resin and the removal of acid labile side-chain protecting groups e.g. *t*-Bu based, Trt and Mtr. The EDT was added as a scavenger for cations derived from cleaved protecting groups, and for the prevention of irreversible back-addition of the detached peptide via methionine and tryptophan to benzyl cations derived from the resin-bound linkage agent. Cleavage of the Mtr protecting group requires prolonged treatment with TFA, but is enhanced by additives. Thioanisole promotes the most rapid removal of Mtr but a purer product was obtained using phenol.[73] Cleavage/deprotection of peptides containing both arginine

and tryptophan have been found to be problematic, as will be considered subsequently. Purging of the cleavage/deprotection mixture with nitrogen before leaving to stand was also found to be advantageous. This appeared to minimise the presence of impurities, less retained on RPHPLC compared to the product, which were probably due to methionine oxidised peptide.

2.1.1.10. Purification Procedures

All peptide analogues were to be subjected to biological testing. Thus, it was imperative that each was rigorously purified. It was common practise to subject the crude peptides to preliminary purification by gel permeation chromatography. This procedure removes low molecular weight impurities, such as those arising from cleaved side-chain protecting groups, residual scavengers, and any fragmented peptides. During cyclisation reactions polymeric material was also successfully separated from the required monomer by this technique. Ion exchange chromatography was attempted on some occasions but was found to offer no significant improvements over procedures already adopted, with higher losses of material resulting. However, preparative RPHPLC was found to be an extremely useful technique. Gradients were employed, formed between 0.1% TFA in water (solvent A) and 0.1% TFA-CH_3CN, 9:1 v/v, (solvent B). The high resolution achieved enabled us to separate diastereomeric peptides. In general, preparative RPHPLC was employed as a final purification, following gel permeation chromatography, although, in some cases, it was sufficient as the sole purification technique. All columns were washed thoroughly before application of the crude samples.

2.1.1.11. Characterisation and Evaluation of Purity

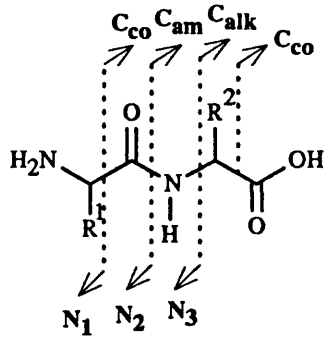
Each compound was characterised by a combination of analytical RPHPLC, t.l.c., amino acid analysis and fast atom bombardment (F.A.B.) mass spectrometry. Selected compounds were further evaluated by ^1H n.m.r. and the reader is directed to Section 2.5. for a discussion of these studies.

The homogeneity of each product was ascertained by a combination of analytical RPHPLC, and t.l.c. in two or more solvent systems, therefore using the resolving powers of both reverse-phase and normal-phase chromatography. A single symmetrical peak by analytical RPHPLC and a

single spot by t.l.c. were both required before submitting the compound to the biological screen.

Analysis of peptides by F.A.B. mass spectrometry was particularly informative, since both molecular weight and structural information could be obtained. The molecular protonated ion, $(M+H)^+$, was observed for each peptide. In some cases, molecular cationised species resulting from addition of Na or K were detected.

For linear peptides, structural information is available from a series of ions formed by fragmentation at specific sites along the peptide backbone, as indicated in **Figure 13.**



N_1 : $H_2N^+=CHR^1$

commonly observed

N_2 : $H_2N\text{-}CHR^1\text{-}C{\equiv}O^+$

common

N_3 : $[\,H_2N\text{-}CHR^1\text{-}CONH_2 + H\,]^+$

common

C_{alk} : $[\,CH_2R^2\text{-}CO_2H + H\,]^+$

rare

C_{am} : $[\,H_2N\text{-}CHR^2\text{-}CO_2H + H\,]^+$

common

C_{co} : $^+O{\equiv}C\text{-}NH\text{-}CHR^2\text{-}CO_2H$

less common

Figure 13

Cleavage may occur in the positions shown, giving rise to ions of the structural types indicated. In general, peptide fragmentations can result from cleavages of NH-C^{α} bond, C^{α}-CO bond or CO-NH bond. In each case the charge can be retained on either the amino or carboxyl terminal position of the peptide. There are also fragment ions associated with various cleavages of side-chain functionalities. However, not all expected fragment ions were seen, and some series predominated over others,

because of favoured fragmentation and/or better charge stabilisation.

Due to the internal cross-link, only limited fragmentation of cyclic peptides occurs. Cleavage to generate ion fragments containing amino acids from within the cross-linked portion would require the cleavage of two bonds, a process much less favourable than the single bond cleavage. It is possible to assign ions to the molecular protonated species and to each of the fragments which result from sequential losses of amino acid residues until the cross-link is encountered. This allows definition of certain amino acids from each terminus to the position of cross-linking.

2.1.1.12. Cyclisation Reactions

Many syntheses involved subsequent cyclisation reactions. When employing oxidising reagents other than molecular oxygen, the use of a nitrogen atmosphere during the cyclisation step was found to suppress dimer and oligomer formation. To this end all oxidation processes were also performed in dilute solution.

Polymeric material was recycled in some cases. Disulphides were customarily reduced with mercaptans e.g. 2-mercaptoethanol. Molar excesses of 20- to 1000-fold are used in order to drive the equilibrium reaction toward the desired products (**Scheme 6**). Simple organic disulphides may be reduced by derivatives of trivalent phosphorus. In particular, tri-*n*-butylphosphine is a potent and specific reducing agent for disulphides, with a non-equilibrium behaviour.[74]

$$R^1\text{-S-S-}R^2 + (R^3\text{-SH})_n \rightleftharpoons R^1\text{SH} + \text{HS-}R^2 + R^3\text{-S-S-}R^3 + (R^3\text{-SH})_{n-2}$$

Scheme 6

The majority of cyclisation reactions were monitored by a quantitative thiol test. This procedure involved the use of Ellman's reagent,[75] 5,5′-dithio*bis*-(2-nitrobenzoic acid), which in aqueous solution at pH 8 reacts with free thiol to form a highly yellow-coloured anion **Figure 14.** Therefore, the initial extent of removal of S-protecting groups and subsequent disappearance of free sulphydryls on cyclisation could be assessed.

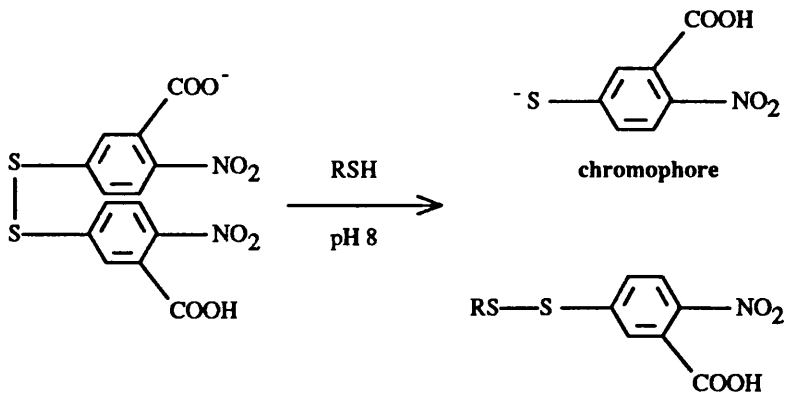


Figure 14

The integrity of the cystine bond is further confirmed by F.A.B. mass spectrometry. It is important that a thiol matrix is not used since this has been observed to cause reduction of the disulphide.

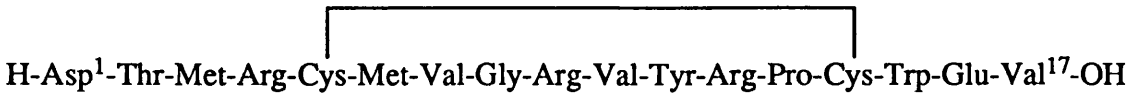
Having outlined the general procedures applied, the syntheses of linear and cyclic analogues of MCH, prepared for "Stage 1" of our study, will be considered in detail.

2.2 SYNTHESES OF LINEAR ANALOGUES OF MCH

The following discussion is concerned with the preparation of linear analogues of MCH.

2.2.1. Sequential Fragments of MCH (1)

To commence the synthetic and structure-activity studies of MCH **(1)**, a series of sequential fragments **(17)-(31)** were prepared, as shown in **Figure 15.** The rationale for performing these syntheses was that the bioassay may reveal particular



(1)

linear MCH(15-17) **(17)**
[Acm-Cys^{14}] linear MCH(14-17) **(18)**
[Acm-Cys^{14}] linear MCH(13-17) **(19)**
[Acm-Cys^{14}] linear MCH(12-17) **(20)**
[Acm-Cys^{14}] linear MCH(11-17) **(21)**
[Acm-Cys^{14}] linear MCH(10-17) **(22)**
[Acm-Cys^{14}] linear MCH(9-17) **(23)**
[Acm-Cys^{14}] linear MCH(8-17) **(24)**
[Acm-Cys^{14}] linear MCH(7-17) **(25)**
[Acm-Cys^{14}] linear MCH(6-17) **(26)**
[Acm-Cys^{5,14}] linear MCH(5-17) **(27)**
[Acm-Cys^{5,14}] linear MCH(4-17) **(28)**
[Acm-Cys^{5,14}] linear MCH(3-17) **(29)**
[Acm-Cys^{5,14}] linear MCH(2-17) **(30)**
[Acm-Cys^{5,14}] linear MCH **(31)**

Figure 15

analogues which show increased potency compared to the precursor, due to the addition of the next amino acid of the sequence. This could indicate the importance of that amino acid for activity. Moreover, such a study may highlight synthetic problems which could be encountered in future work.

The syntheses proceeded relatively smoothly. Valine, the first amino acid of the sequence, was introduced by the usual two batch coupling procedure, employing the active ester derivative Fmoc-Val-OPfp (with the normal amount of DMAP), for 4 h and 15 h respectively. For these initial syntheses, neither the quantitative ninhydrin colour test or the quantitative Fmoc determination had been fully developed in our hands. Thus, no quantification of the extent of resin acylation was obtained, and the synthesis proceeded in the customary manner, adding each Fmoc-amino acid derivative until the complete MCH sequence had been assembled. After each deprotection stage from the tripeptide **(17)** onwards, a portion of the peptidyl-resin was removed to isolate each fragment of the series. Fmoc-amino acids were introduced using the Pfp ester derivative except in the case of Fmoc-Arg(Mtr) and Fmoc-Thr(*t*-Bu) where the Dhbt ester was employed. The Acm protecting group was used for the cysteine residue. Each coupling was complete after the normal 25 min period, except for Pro^{13} (120 min), Arg^{9} (100 min) and Cys^{5} (120 min).

A modified cleavage/deprotection procedure was used for the peptidyl-resins of compounds **(17)** - **(19)**, where a mixture of anisole and EDT was found to be the most effective in scavenging for tryptophan, with a period of only 2 h required to cleave the peptide from the resin and remove the O-*t*-Bu protecting group from the glutamic acid residue.

Cleavage/deprotection of the peptidyl-resin samples of compounds **(20)** - **(31)** was carried out following the usual protocol, a period of 7 h being the optimum in each case. It was found that removal of the Mtr protecting groups did not appear to be entirely complete after the 7 h period (as indicated by analytical RPHPLC), and it appeared that longer periods were required as the number of Arg(Mtr) residues increased. However, if cleavage/deprotection was allowed to proceed for extended periods, then formation of a by-product occurred, to the detriment of the fully deprotected peptide. In each case, the by-product was more retained on RPHPLC compared to the desired product. A similar observation was made by Eberle and co-workers[11] during their synthesis of MCH. Using identical protecting groups to those employed in our case, the MCH peptidyl-resin was treated with TFA in the presence of anisole and EDT for 5 h. The crude material obtained after cleavage was found to contain a contaminant possessing anomalous u.v. absorption, which was thought to be a

by-product of cleavage of the Mtr protecting group and/or containing a modified tryptophan residue.

After work-up, the yield of each crude peptide was uniformly low. Purification was carried out successfully by preparative RPHPLC alone, to afford pure samples of each compound **(17)**-**(31)**, in yields varying from 14-26%. It is our opinion that these poor yields are due to low resin coverage on the initial esterification reaction with valine, despite employing extended coupling periods. Difficulties in acylation reactions involving this sterically hindered amino acid have been reported many times in the literature. It has since been shown that the esterification proceeds in higher yield when the more reactive symmetrical anhydride of Fmoc-Val is employed (see Section 2.2.4.1.).

2.2.2. [Acm-Cys^{5,14},Phe^{11}] linear MCH (32)

Modification of the Tyr^{11} residue of MCH results in reduced activity, as both [NO_2-Tyr^{11}]MCH[8] and [I-Tyr^{11}]MCH[12] had lower potencies compared to the parent peptide; thus emphasising the importance of this residue. This finding prompted our synthesis of [Acm-Cys^{5,14},Phe^{11}] linear MCH **(32)**, where the Tyr^{11} residue is substituted by Phe to test the effect on activity of the absence of the phenolic OH. A similar substitution was carried out in a structure-activity study of oxytocin.[63]

The analogue **(32)** was prepared in a parallel synthesis with that of the compounds **(17)**-**(31)** (as considered earlier in section 2.2.1.), by the addition of Fmoc-Phe-OPfp in place of Fmoc-Tyr(*t*-Bu)-OPfp at the appropriate point. A normal coupling time ensued and cleavage/deprotection of the peptidyl-resin proceeded as usual with an optimum period of 7 h. Purification of the crude peptide was carried out by preparative RPHPLC, using 10 μ microparticulate porous silica packing, as opposed to the usual 5 μ particle size, with the intention of improving product yield. However, the yield was again disappointing at only 14%.

2.2.3. *[Acm-Cys^{14}] linear MCH(9-14) (33) and [Acm-Cys^{14}] linear MCH(10-14) (34)

The importance of the Tyr^{11} residue has already been implied. It is also a possibility that the residues in the region of Tyr^{11} may be important for molecular activity. Thus, the fragments [Acm-Cys^{14}] linear MCH(9-14) (33) and [Acm-Cys^{14}] linear MCH(10-14) (34) containing residues on either side of Tyr^{11},were prepared.

H-Arg^{9}-Val-Tyr-Arg-Pro-Cys^{14}(Acm)-OH

(33)

H-Val^{10}-Tyr-Arg-Pro-Cys^{14}(Acm)-OH

(34)

Synthesis was carried out in the usual manner, by employing the customary protecting groups, with Cys^{14} protected as the Acm derivative. Hence pure samples of each compound (33) and (34) were obtained for the biological assay.*

2.2.4. Linear MCH(1-4,15-17) (35) and linear MCH(1-4, Aha,15-17) (36)

To our knowledge, no structure-activity study has yet been carried out on the exocyclic fragments of MCH alone. We were therefore interested in gaining some understanding of their importance for activity/binding.

The C-terminal tripeptide, linear MCH(15-17) (17), has already been prepared and we next turned our attention to the syntheses of the joined N-terminal and C-terminal exocyclic sequences, linear MCH(1-4,15-17) (35) and linear MCH(1-4,Aha,15-17) (36).

Thus in (35), the exocyclic sequences are directly connected. In (36),

H-Asp[1]-Thr-Met-Arg[4]-Trp[15]-Glu-Val[17]-OH

(35)

H-Asp[1]-Thr-Met-Arg[4]-Aha-Trp[15]-Glu-Val[17]-OH

(36)

Aha = -NH-$(CH_2)_6$-CO-

however, the inserted Aha unit (7-aminoheptanoic acid, -NH-$(CH_2)_6$-CO-) spaces the terminal sequences at the same calculated distance apart as in the native peptide (**Figure 16**).

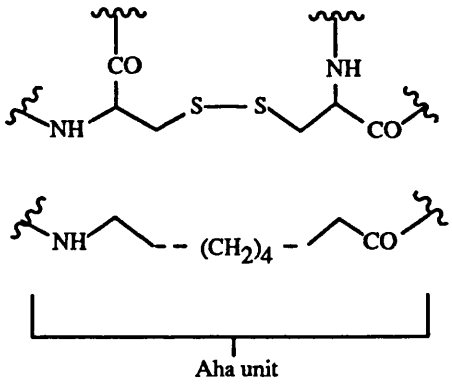


Figure 16

2.2.4.1. Linear MCH(1-4,15-17) (35)

The synthesis proceeded relatively smoothly. The first Fmoc-amino acid to be coupled to the resin was valine. Experience gained in the synthesis of the sequential linear fragments, (17)-(31) and (32), had indicated that use of the symmetrical anhydride of Fmoc-Val, as opposed to the Pfp ester, may result in higher resin coverage. Thus, two batches of the freshly prepared Fmoc-Val anhydride were employed and each recirculated for 50 minutes. The quantitative ninhydrin colour test indicated that the esterification reaction had proceeded in 91% yield. Therefore, it appears

that the symmetrical anhydride is the acylating reagent of choice for direct attachment of Fmoc-Val to the resin. Low coverage resulted even after periods of 4 h and 15 h when using the Pfp ester.

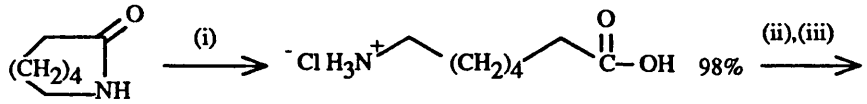
When coupling Fmoc-Arg(Mtr), as its pre-formed anhydride, the qualitative Kaiser test indicated that the acylation reaction was incomplete even after a 50 minute recirculation period. It is thought that the anhydride of this side-chain protected derivative is unstable,[76] and does not persist in the reaction medium long enough when the acylation becomes sluggish for steric or other reasons. However, complete acylation was achieved after the addition and recirculation (50 min) of a fresh batch of Fmoc-Arg(Mtr) anhydride.

Cleavage/deprotection was carried out in the customary manner with an optimum period of 7 h. The crude peptide was obtained in good yield (89%), hence validating the use of Fmoc-Val anhydride for the initial esterification reaction with the resin bound active sites. Following the usual purification procedures, the pure peptide was obtained in 33% yield.

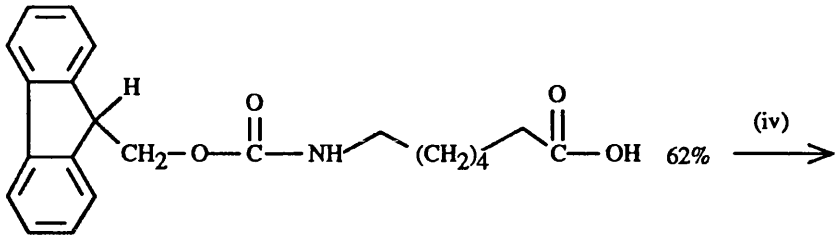
2.2.4.2. Linear MCH(1-4,Aha,15-17) (36)

In order to incorporate the Aha unit it was necessary to synthesise a derivative that was suitably functionalised for use in solid phase assembly. Thus the compound pentafluorophenyl N^{α}-(9-fluorenylmethyloxycarbonyl)-7-aminoheptanoate (**37**) was targeted. Preparation was carried out in three steps according to **Scheme 7**.

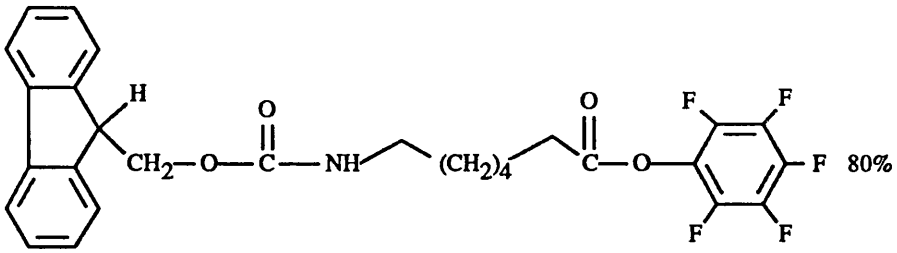
The hydrochloride salt of 7-aminoheptanoic acid (**38**) was first obtained by the acid catalysed hydrolysis of 2-azacyclooctanone. Neutralisation of (**38**) with aqueous Na_2CO_3 was followed by formation of the Fmoc derivative (**39**) by reaction with 9-fluorenylmethyl chloroformate[77] in the presence of aqueous Na_2CO_3. The product was observed to precipitate out in the reaction mixture. Spectroscopic data indicated that the Fmoc group had been successfully incorporated, though the carboxylic acid proton of (**39**) remained undetected by ^1H n.m.r. in DMSO. Literature precedents validate the use of alternative reagents to introduce the Fmoc functionality since 9-fluorenylmethyl chloroformate has been shown to afford the product accompanied by the undesired, presumably mixed anhydride mediated, formation of significant amounts



(38)



(39)



(37)

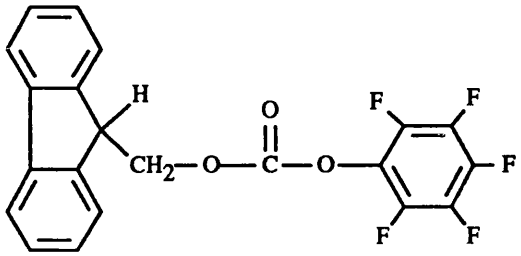
(i) 2*N* HCl, reflux, 1.5 h (ii) 10% aq. Na_2CO_3, dioxan (iii) Fmoc-Cl, dioxan ; 0°C, 1 h ; RT, 18 h (iv) Pfp-OH, DCC, DMF-EtOAc ; 0°C, 1 h ; RT, 1 h

Scheme 7

(3-20%) of Fmoc-dipeptide.[78] The application of 9-fluorenylmethyl carbonoazidate[78a] and mixed carbonates[78b] have been suggested as side-reaction-free alternatives. In our case however, 9-fluorenylmethyl chloroformate performed satisfactorily since formation of Fmoc-dipeptide could not be detected by chromatographic or spectroscopic means.

The Pfp ester derivative (37) was formed following the procedure of Kisfaludy et al,[79] by the addition of pentafluorophenol and DCC as the activating species. In this reaction it was imperative that DMF used in the solvent system was freshly distilled to remove unwanted dimethylamine which could cause undesired cleavage of the Fmoc group. Since C-F carbons of the Pfp group are strongly coupled in ^{13}C n.m.r. spectroscopy, a semi-quantitative comparison with a sample of Fmoc-Leu-OPfp, by ^{19}F n.m.r., was used to show the Pfp group to be present as required. In addition, the characteristic Pfp ester i.r. absorption at ν_{max} 1780 cm^{-1} was also detected.

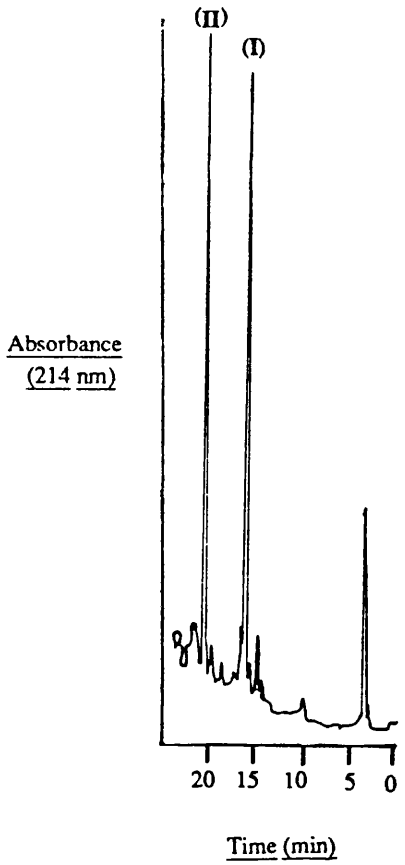
It is interesting to note that Fmoc-amino acid pentafluorophenyl esters can be prepared by a double utilisation of mixed carbonates. A useful reagent is 9-fluorenylmethyl pentafluorophenyl carbonate[80] **(40)**, which effects efficient side-reaction-free-introduction of the Fmoc protecting group and subsequent preparation of the Pfp derivative.



(40)

Having successfully synthesised Fmoc-Aha-OPfp **(37)**, the synthesis of linear MCH(1-4,Aha,15-17) **(36)** was performed in parallel with that of linear MCH(1-4,15-17) **(35)**. Fmoc-Aha-OPfp was added at the appropriate point and successful acylation of Trp^{15} was achieved after the standard 25 min period. However, deprotection of the Aha amino group did not take place during the usual ten min reaction time, as no deprotection peak was seen and in addition, the Kaiser test showed no development of the blue colour characteristic of free amine. Exposure to 20% piperidine-DMF for 15 h was required to achieve cleavage. There is no obvious explanation for this phenomenon of reduced rate of cleavage. It is possible however that intra-chain and/or inter-chain interactions may have occurred. Removal of all the subsequent Fmoc-groups was normal.

As discussed earlier in relation to the cleavage/deprotection of the peptidyl-resin of compounds **(17)**-**(31)**, a cleavage period longer than the stipulated 7 h resulted in the formation of a by-product. During a trial cleavage of the peptidyl-resin of **(36)**, the reaction was allowed to proceed for an extended 36 h period, after which time two peaks, (I) and (II), of approximately equal heights were detected as shown in **Figure 17**.

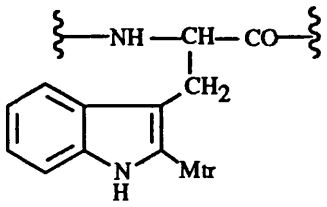


Synthesis of (36) : analytical RPHPLC of the crude product following prolonged cleavage / deprotection , showing the desired peptide peak (I) , and the by-product peak (II) .

Figure 17

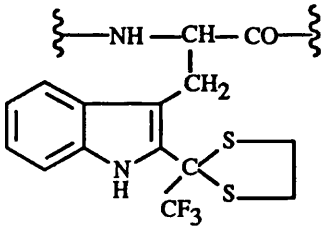
The observed peak (I) was thought to be due to the desired product and peak (II) to the by-product. It was our intention to identify this by-product and to this end it was isolated by preparative RPHPLC and analysed by mass spectrometry (F.A.B.). No separation was effected by the preceeding gel permeation chromatography. A major fragment at m/z 1237 was detected.

One possibility considered in the case of compounds **(17)-(31)** was that the by-product could be an analogue in which tryptophan has been modified by the cleaved Mtr group **(41)**. However, we could suggest no such



(41)

analogue that would fit the mass spectrometry finding. It came to our attention that Sieber[81] had carried out studies on the effect of scavengers on the modification of tryptophan residues during acidolysis of Mtr groups. The formation of the lipophilic by-product (**41**) was found to occur on treatment of a model peptide with TFA-H_2O, but was minimised by the addition of EDT. However, if the cleavage was left in the presence of this scavenger for extended periods then a dithioketal product (**42**) was formed, which was ultimately the sole product. Thus, it appears that the by-product obtained in our synthesis could be attributed to an analogue where tryptophan has been modified in this fashion during the prolonged cleavage period. Such a by-product would give rise to an $(M+H)^+$ peak at m/z 1237, as we observed.



(42)

Removal of the Mtr group from arginine in the presence of the sensitive tryptophan residue is therefore problematic, where Mtr requires more vigorous conditions for cleavage compared to the other acid-labile protecting groups employed. The nature and extent of tryptophan modification is dependent on the scavengers used and the length of the reaction period, thus these factors would have affected the type of by-products observed in the syntheses of (**17**)-(**31**) and (**32**).

Our experiment has therefore confirmed that it is imperative to perform trial cleavages and to keep the reaction time to a minimum in the presence of sensitive residues. Hence we were able to obtain the desired product successfully, adopting a 7 h optimum cleavage period, with the normal cleavage/deprotection mixture. Pure material was obtained in 39% yield with the presence of the spacer unit readily confirmed by F.A.B. mass spectrometry, m/z 1064 $(M+H)^+$.

2.2.5. [Acm-Cys^{5,14}] linear MCH(5-14) (43)

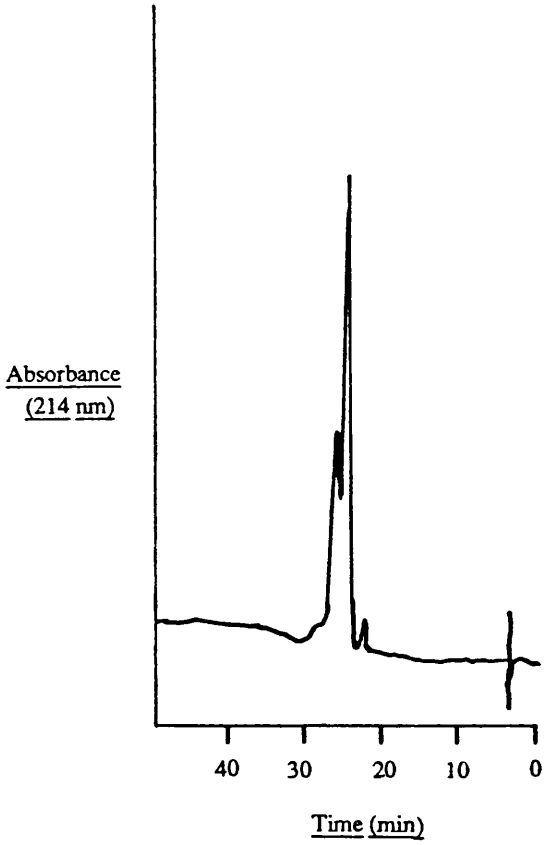
Having completed syntheses of exocyclic fragments of MCH, a study of the substructural unit comprising residues 5-14 of the cyclic portion of the peptide was undertaken. The disulphide ring-closed fragment has been prepared and will be considered subsequently, however, the linear form was also of interest. A comparison of the relative potencies of each derivative may give an indication of the importance of the disulphide bond for activity.

The fragment [Acm-Cys^{5,14}] linear MCH(5-14) **(43)** was thus targeted, where the cysteine residues were permanently protected as the Acm derivatives. Synthesis was attempted following the usual procedures. For attachment of the first residue, cysteine, to the resin, the preformed symmetrical anhydride procedure was adopted, using the normal amount of DMAP. Fmoc-Cys(Acm) anhydride was therefore prepared and was seen to precipitate during its formation. The synthesis then appeared to proceed in a satisfactory manner.

H-Cys^5(Acm)-Met-Val-Gly-Arg-Val-Tyr-Arg-Pro-Cys^{14}(Acm)-OH

(43)

The peptide does not possess a tryptophan residue and thus cleavage/deprotection of the peptidyl-resin was not accompanied by the associated side-product formation. The reaction could be allowed to proceed for longer periods with an optimum time of 15 h. However, to our dismay, analysis of the crude product by RPHPLC revealed two overlapping peaks in an approximate 2:1 ratio (**Figure 18**). These remained unseparated following gel permeation chromatography and thus



Synthesis of (43) : analytical RPHPLC of crude product showing two overlapping peaks .

Figure 18

small amounts of each component were isolated by preparative RPHPLC. Identical mass spectra (F.A.B.) analyses, compatible with the expected product, were obtained in each case and it is postulated therefore that the two compounds are diastereomers.

The mixture was later cyclised and the two products separated by preparative RPHPLC. They were indistinguishable by amino acid analysis and F.A.B. mass spectrometry and a comparison of their ^1H n.m.r. spectra suggests that they are cyclic MCH(5-14) (7) and its [DCys^{14}] analogue (44).

H-Cys^5-Met-Val-Gly-Arg-Val-Tyr-Arg-Pro-Cys^{14}-OH

(7)

H-Cys[5]-Met-Val-Gly-Arg-Val-Tyr-Arg-Pro-DCys[14]-OH

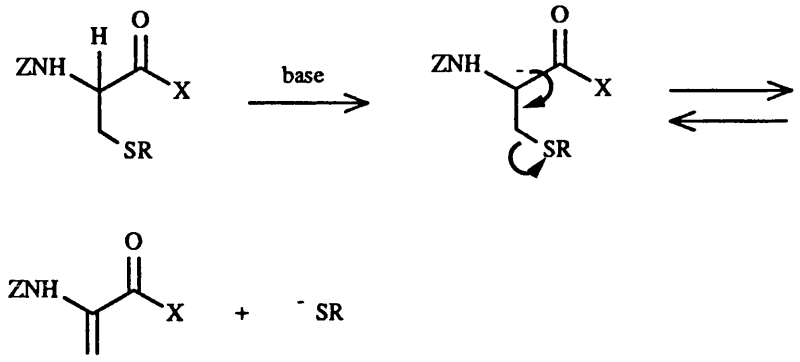
(44)

Epimerisation of urethane-protected amino acids by DMAP was shown to be a significant side reaction during attachment of the first amino acid to the resin.[27] Therefore, it appears that racemisation of Cys^{14} has occurred during this process where the coupling was carried out in the presence of DMAP, known to induce racemisation by proton abstraction.

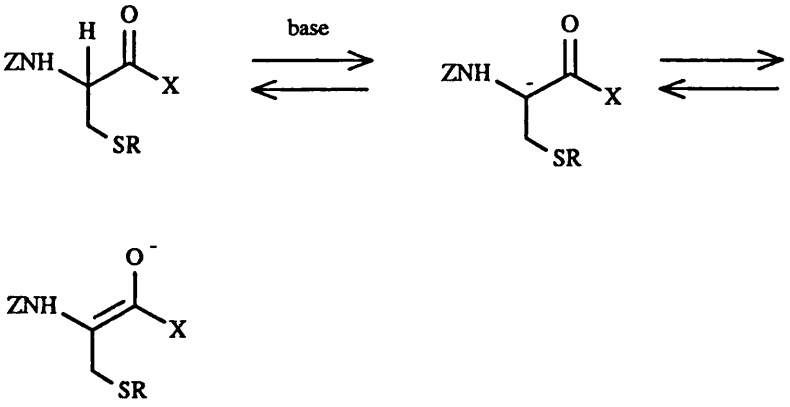
Amino acids such as cysteine, with electronegative substituents in the β-position, are particularly prone to racemisation, where the lability of the C^{α} proton is enhanced by inductive stabilisation of the resulting anion. Two different mechanisms can be envisaged for this process and are outlined in **Scheme 8.** One route (a) is via β-elimination-readdition of the side chain.[82] The second route (b) is by direct abstraction of the α-proton, without elimination, known as the isoracemisation or "conducted tour" mechanism proposed by Kovacs.[83] Moreover, contrary to long held beliefs, oxazolone formation and hence racemisation of urethane protected α-amino acids is indeed possible.[84]

It is important to add DMAP to the resin after the addition of the Fmoc-amino acid derivative, during attachment of the first residue, in order to minimise racemisation. In addition, shortening of the coupling time, and particularly reducing the amount of DMAP added, were found by Atherton et al[27] to be important strategies. Moreover, these workers showed there was no evidence for racemisation of the resin-bound species when an active ester coupling was employed (under the same conditions used for the symmetrical anhydride).

(a) β - elimination-readdition of the side chain



(b) direct C^{α}- proton abstraction



Scheme 8

In our subsequent attempt to obtain the unracemised product (**43**) we used only 0.025 molar equivalents of DMAP, and the usual anhydride method was replaced by a double Pfp ester couple, each of 4 h duration. The synthesis then proceeded in the customary manner having achieved satisfactory acylation of the resin. All couplings except that of Arg^9 (50 min) were complete after the customary 25 min period. Cleavage/deprotection, with an optimum period of 15 h, furnished the crude material which now gave rise to virtually a single peak on analytical RPHPLC. Thus, it appears that no detectable racemisation has occurred and hence procedures adopted to obtain the single diastereomer have been successful. Following purification, the homogeneous product [Acm-$Cys^{5,14}$] linear MCH(5-14) (**43**) was obtained in 69% yield.

Although racemisation of urethane-protected amino acid derivatives may not be a significant problem in the normal processes of solution or solid phase peptide synthesis, serious consideration should be made in special circumstances, particularly when using basic catalysts, as in attachment of the first residue to the resin. Racemisation may be circumvented by employing one of the available methods for first residue attachment where the use of DMAP is avoided. Slight racemisation is less problematic provided that the single unwanted diastereomer can be separated.

2.2.6. [Acm-Cys^{5,14},Phe^{11}] linear MCH(5-14) (45)

To further test the importance of the Tyr^{11} residue, the Phe analogue of **(43)**, [Acm-Cys^{5,14},Phe^{11}] linear MCH(5-14) **(45)** was prepared. Synthesis was carried out in parallel with that of **(43)**, substituting Fmoc-Phe-OPfp (coupling time 25 min) for Fmoc-Tyr(*t*-Bu)-OPfp at the appropriate point. Following cleavage/deprotection (optimum period 15 h), and subsequent purification, the homogeneous product was obtained in 65% yield.

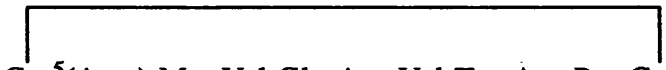
2.3. SYNTHESES OF CYCLIC ANALOGUES OF MCH

Having accomplished the preparation of various linear fragments of MCH, attention was turned to the syntheses of cyclic analogues. Such compounds are inherently of interest, due to their limited conformational freedom.

2.3.1. *[Acm-Cys^{5,14}] cyclic MCH(5-14) (46)

As part of our investigation into the importance of the disulphide bond for activity, an analogue comprising the residues 5-14 of the ring portion was required, where cyclisation is effected by a bond other than disulphide. The homodectic peptide [Acm-Cys^{5,14}] cyclic MCH(5-14) **(46)**, where ring closure was achieved via an amide bond between the two Acm protected Cys^{5,14} residues, was targeted.

Studies of the importance of the disulphide group for biological activity in other hormones have been carried out, where various carba-analogues of hormones which ordinarily contain a disulphide bridge (e.g.

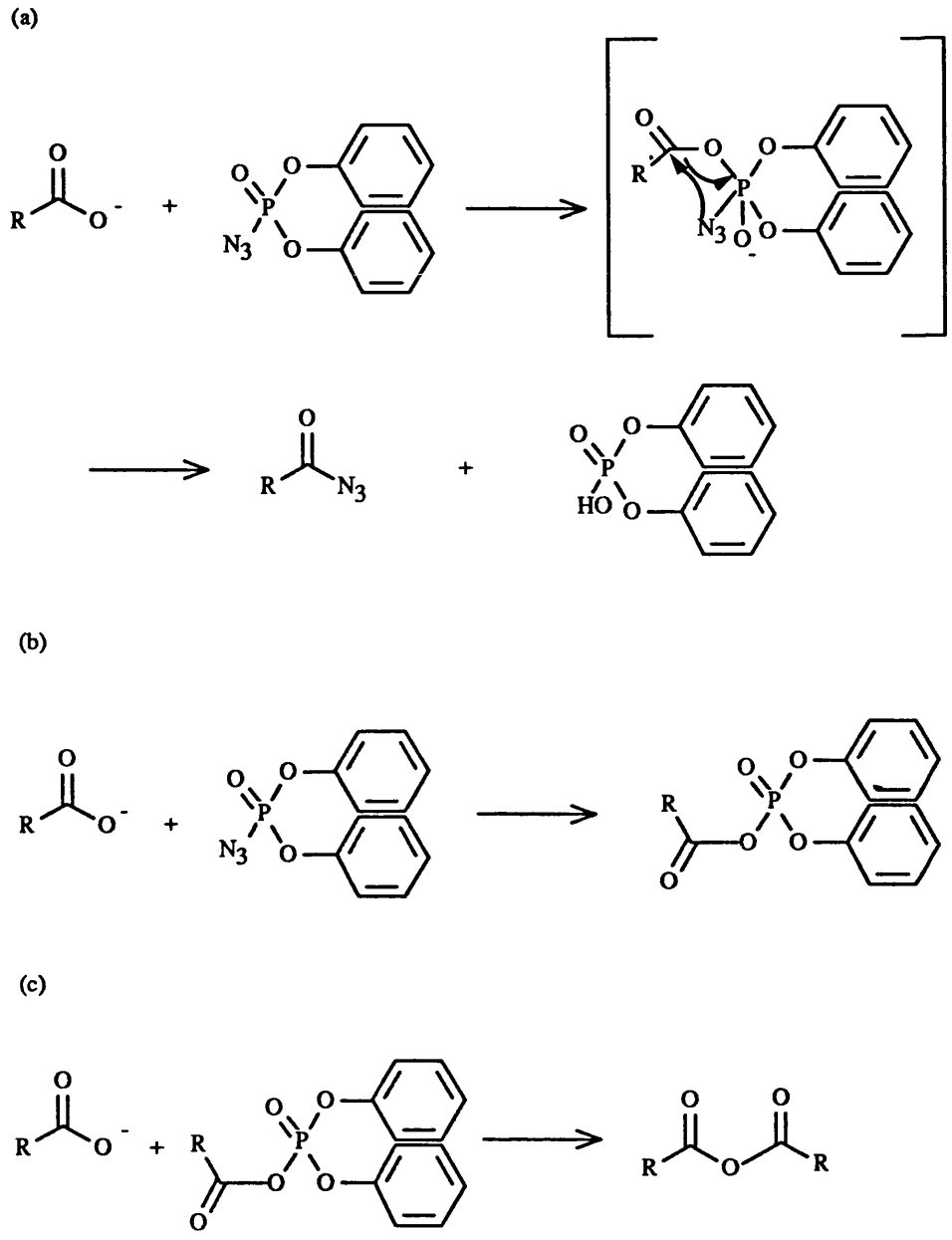


Cys[5](Acm)-Met-Val-Gly-Arg-Val-Tyr-Arg-Pro-Cys[14](Acm)

(46)

oxytoxin), were synthesised.[85] The final cyclisation was effected via an amide bond, for which various methods are available, generally based on the well known coupling protocols of peptide synthesis e.g. the azide, mixed anhydride, active ester and DCC procedures.

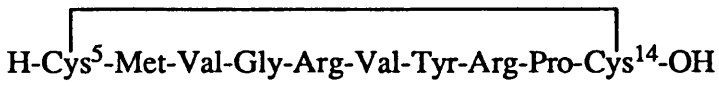
It was found that diphenylphosphoryl azide,[40a] with added HOBt, provided efficient activation for ring closure. The cyclisation was accomplished in high yield (42%), both with prior or subsequent, side-chain deprotection. In the latter case the synthesis was performed using the 4-hydroxymethyl-3-methoxyphenoxyacetic acid handle (**11**), providing lability of the peptide-resin linkage to 1% TFA. This yielded the fully protected peptide with a C-terminal caboxylic acid. The reaction may involve (a) acyl azide formation *in situ*, but also seems likely to proceed via (b) mixed diphenylphosphinic or (c) symmetrical anhydrides. Moreover, the added HOBt may not only be effective in reducing racemisation, but could also be involved in the reaction by formation of the HOBt ester. The various reactive intermediates (a)-(c) are outlined in **Scheme 9.**



Scheme 9

2.3.2. Cyclic MCH(5-14) (7)

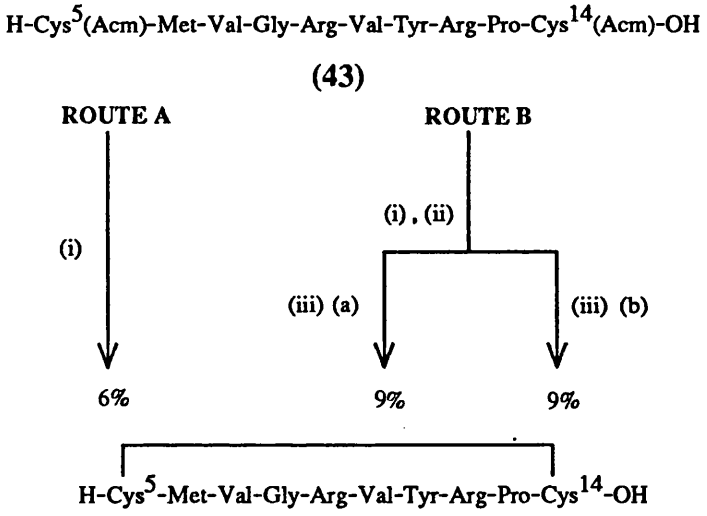
Having synthesised the linear and amide cyclised compounds containing only residues of the ring portion of MCH, we turned our attention to the preparation of cyclic MCH(5-14) (**7**), where cyclisation is achieved by a disulphide bond, as in the native molecule. These three analogues were required in order that a complete study could be carried out on both the importance of the 5-14 subunit and the cystine bridge for



(7)

activity.

In attempts to synthesise **(7)**, various methods for disulphide bond formation were tried, in order to find a suitable procedure that could also be readily applied in future syntheses of other cyclic analogues. Reactions were carried out on the corresponding linear compound where both the cysteines were initially protected as either the Acm **(Scheme 10)**, or Trt **(Scheme 11)**, derivatives.



ROUTE A

(i) I_2, 90% aq. AcOH, RT, 0·5 h

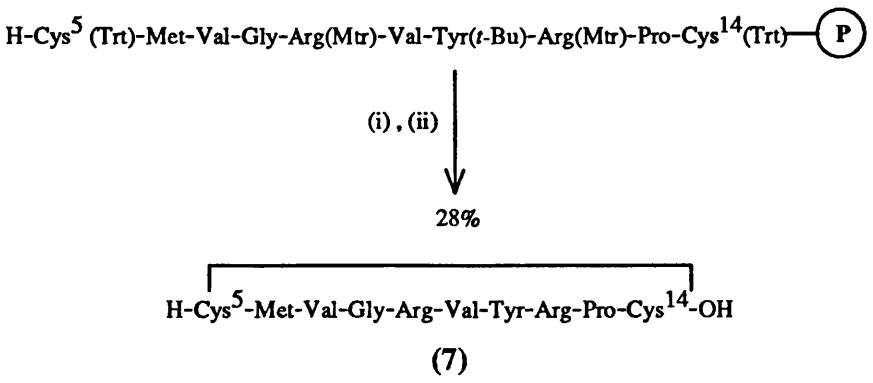
ROUTE B

(i) $Hg(OCOCH_3)_2$, pH 4, RT, 4 h (ii) H_2S

(iii) (a) [O], pH 7, RT, 15 h

(iii) (b) $K^+ Fe(CN)_6^{2-}$, pH 7, RT, 2 h

Scheme 10



Scheme 11

2.3.2.1. Iodine Mediated Oxidation of the [Acm-Cys^{5,14}] Protected Peptide

The analogue [Acm-Cys^{5,14}] linear MCH(5-14) **(43)** was already in hand, thus deprotection and simultaneous disulphide bond formation was attempted by treatment with iodine.[64]

The reaction was carried out following essentially the same procedure as that described by Minamitake et al.[86] The solvent of choice was 90% aqueous acetic acid solution. Studies carried out by Kamber[87] indicated favourable rates in aqueous acetic acid solution, where the addition of water enhances the reaction. Dilute hydrochloric acid was added in an attempt to safeguard against iodination of tyrosine, though this is considered to be much less severe in the solvent system adopted. It is still necessary however to keep the reaction time as short as possible. Excess iodine (50 equivalents in 90% aqueous acetic acid) was employed, to which the peptide solution was added. It has been suggested that the reverse mode of addition may result in larger quantities of polymeric material.

Following a 30 minute reaction period, excess iodine was extracted with carbon tetrachloride, but following lyophilisation, traces of iodine still remained which were quenched by the addition of aqueous ascorbic acid. Purification was attempted by ion exchange chromatography, since this may enable efficient separation of any remaining Acm protected peptide from the desired product. However, only non-volatile buffers and a salt gradient were effective in eluting peptidic material, and subsequent desalting methods proved unsuccessful. The usual purification procedures

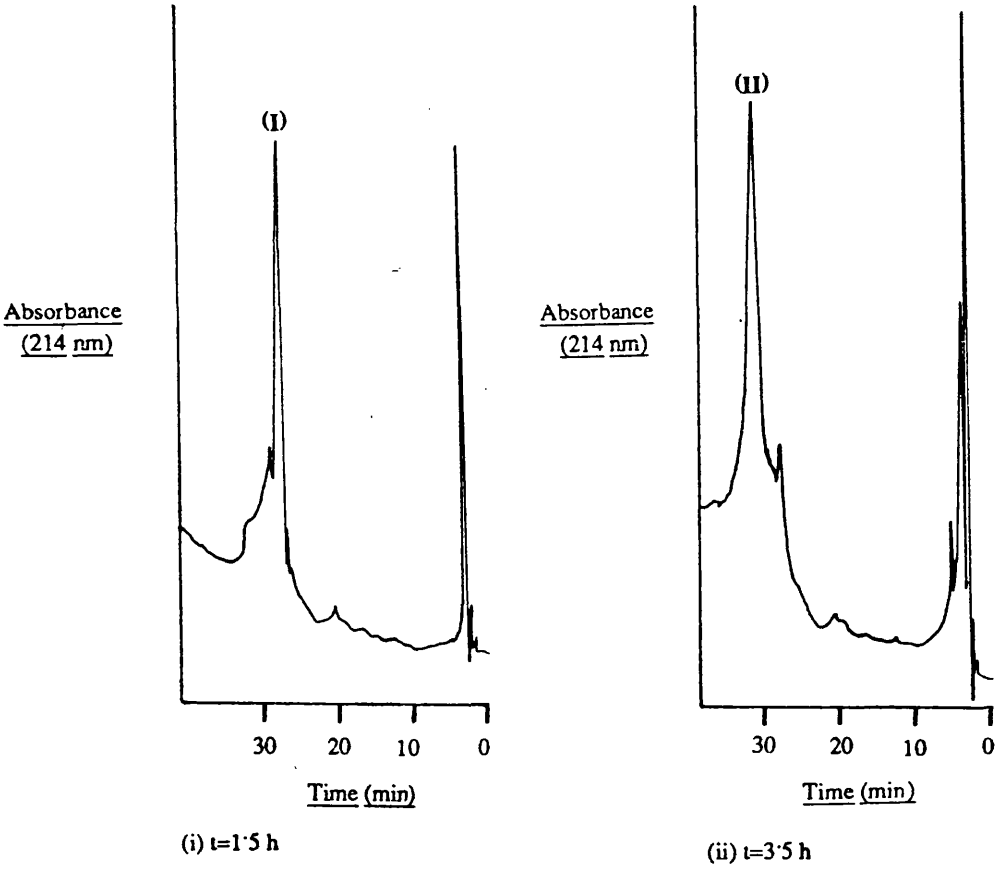
of gel permeation chromatography, followed by preparative RPHPLC, were employed. The product obtained was shown by F.A.B. mass spectrometry to correspond to [O-Met^6] cyclic MCH(5-14) **(47)**, where methionine has been oxidised to the corresponding sulphoxide. There was no evidence for Acm protected peptide. It should be noted that a combination of ammonium iodide and dimethyl sulphide in TFA has been found to be effective in reduction of methionine sulphoxide to methionine, without affecting the cystine bond.[88]

The undesired oxidation of methionine could be suppressed by performing the reaction under nitrogen, in purged solutions, with addition of methionine as a scavenger. Isolation of the product from this system was difficult, particularly in the separation of peptide from involatile salts, and recovered amounts of pure product never exceeded 6% yield. Alternative methods for disulphide bond formation were therefore investigated. In later syntheses of other analogues, we have found iodine oxidation to proceed successfully using a slightly modified procedure.

2.3.2.2. Cleavage of Acm Protecting Groups with Mercuric Acetate

Heavy metal salts e.g. of silver and mercury, have been used to remove the Acm protecting group. This method is of value because reaction conditions are mild. The formation of disulphide is then accomplished in a separate oxidation step.

Cleavage of the Acm protecting group from [Acm-Cys^{5,14}] linear MCH(5-14) **(43)** was effected by exposure to aqueous mercuric acetate solution at pH 4 for 4 h.[89] The cleavage proceeds more slowly both above and below pH 4; at higher pH mercuric ion is precipitated as HgO. The reaction was carried out under nitrogen to prevent oxidation of methionine and premature disulphide formation. Monitoring by analytical RPHPLC, at hourly intervals, showed an initial peak (I), due to the starting material, which diminished with time as a broad peak (II), corresponding to the mercuric salt of the peptide, appeared (as illustrated in **Figure 19**). After 4 h, only the product peak was evident. Removal of Hg^{2+} was accomplished by addition of hydrogen sulphide. Scrupulous removal of excess H_2S, by a nitrogen purge, was subsequently required to prevent the formation of



Synthesis of (7) : analytical RPHPLC trace showing the cleavage reaction of (43) with Hg^{2+} after : (i) 1·5 h and (ii) 3·5 h .

Figure 19

S-sulphonates resulting from the oxidation of H_2S to SO_2. Having removed the precipitated mercuric sulphide, treatment with 2-mercaptoethanol-acetic acid ensured total removal of Hg^{2+} and reduction of any preformed disulphide. Gel permeation chromatography was performed under nitrogen in presence of the reducing agent. The crude dihydro peptide was isolated in 44% yield. A portion of the crude material was rapidly purified by preparative RPHPLC. The assay using Ellman's reagent determined a 61% yield of free thiol groups.

Mercuric acetate was therefore successful in causing cleavage of the Acm protecting groups. The reaction was readily monitored by analytical RPHPLC, unlike in the iodine oxidation method. However, low yield of product was again problematic. Some losses may have occurred by adsorption of the peptide on mercuric sulphide. Alternatively, treatment with excess 2-mercaptoethanol and removal of mercurimercaptoethanol by gel permeation may be the method of choice.

The crude dihydro peptide was used without further purification in the following cyclisation reactions, mediated by (a) aeration, and (b) potassium ferricyanide.

(a) Air Oxidation

Molecular oxygen[90] has been used to synthesise disulphide-cyclised peptides from the corresponding disulphydryl compound in dilute aqueous solutions. Steps can be taken to minimise the production of polymeric material. The solution should be degassed by a nitrogen purge before leaving to stand. The reaction is best carried out in a conical flask such that the surface area in contact with the atmosphere is minimised. These precautions allow the slow diffusion of oxygen through the system and hence favour the production of monomer over dimer and oligomer.

The crude dihydro peptide, linear MCH(5-14) **(48)**, was thus subjected to air oxidation in degassed, neutral buffered water in a narrow necked flask open to the atmosphere for 15 h at RT. During this period a small amount of precipitate developed, presumably polymeric material. Following confirmation of the absence of free sulphydryls, the crude material was purified to yield the desired product **(7)** in only 21% yield (from the crude disulphydryl peptide). Gel permeation chromatography had indicated a relatively large quantity of polymeric material to be present.

(b) Potassium Ferricyanide Oxidation

Formation of intra-molecular disulphide bonds from sulphydryl group containing peptides can be accomplished in dilute, neutral solutions, by potassium ferricyanide. This oxidising agent has proved to be effective in the preparation of the cystine bond of MCH,[10] and related analogues, from the corresponding dihydro peptides. Potassium ferricyanide does not appear to oxidise thiols beyond the disulphide stage or to affect the phenolic function of tyrosine.

Thus the crude dihydro peptide, linear MCH(5-14) **(48)**, as a dilute aqueous solution in degassed, neutral buffer, was treated with an aqueous solution of potassium ferricyanide. The "end-point" of the reaction is indicated by a permanent yellow colour of the solution due to the presence

of excess ferricyanide reagent. However, since a faint yellow colour appeared in the solution after the addition of only a small portion of the theoretical amount of ferricyanide required, a small excess (10%) of the reagent was employed to ensure complete absence of traces of thiol. Work-up proceeded after a 2 h reaction period and following purification, a product homogeneous to that from the air oxidation process was obtained in 22% yield (from the crude disulphydryl peptide). A large portion of polymeric material was again separated by gel permeation chromatography.

Our results indicate that both air and ferricyanide are effective in causing oxidative ring closure. The yield of product from each process was very similar (*ca.* 22%). However, the reaction is faster when using ferricyanide, the more potent oxidising agent, though formation of polymeric material is more likely. It is vital that the ferricyanide reaction is performed under nitrogen in order to suppress the production of dimer and oligomer.

The yield of cyclised product from the Cys(Acm) protected peptide was disappointing in each case, being *ca.* 9%. It is our perception that some losses have occurred by adsorption of the peptide onto mercuric sulphide. Moreover, it is likely that premature disulphide bond formation has occurred during handling, before the controlled oxidation processes were employed. This resulted in production of the polymeric by-product as was detected by gel permeation chromatography. It has been found that the linear dihydro peptide (**48**) polymerises on standing. The oxidation step must therefore be employed as soon as this compound is isolated.

2.3.2.3. Acidolytic Treatment of [Trt-Cys^{5,14}]-Peptidyl Resin followed by Air Oxidation

Our previous attempts at the synthesis of cyclic MCH(5-14) (**7**) have been successful, yet yields have remained low. An alternative method was still desirable and to this end a relatively simplistic approach was adopted, where cysteine protecting groups were more conveniently removed **(Scheme 11)**.

The S-Trt group in both cysteine and cysteine peptides is readily cleaved at RT by TFA.[70] Thus, we employed Trt protected cysteines in the synthesis, such that the trityl groups could be removed during TFA

mediated cleavage/deprotection of the peptidyl-resin. Oxidation by aeration or ferricyanide of the disulphydryl peptide obtained should provide an accessible route to **(7)**.

The [Trt-Cys^{5,14}]-peptidyl-resin was therefore required. In order to suppress racemisation, Fmoc-Cys(Trt) was esterified to the resin by employing the Pfp ester couple in the presence of the reduced amount of DMAP (0.025 molar equivalents). It has been suggested that the S-Trt derivative of cysteine is highly lipophilic and conflicts with the generally polar character of the Fmoc-polyamide system.[11] Thus, three batches of reactants were employed, applying recirculation periods of 2 h, 15 h and 2 h respectively. The quantitative ninhydrin assay indicated a reasonable resin coverage of 74%. The synthesis was then continued in the usual manner, with all couplings complete after 25 min except for Val^{10} (60 min), Arg^{9} (60 min) and Val^{7} (90 min).

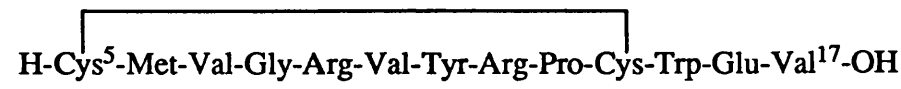
The removal of S-Trt by TFA is an equilibrium reaction, which is displaced during the removal of TFA so that retritylation of the SH group is favoured. It has been found[70] that the reaction conditions and work-up must therefore be carefully controlled. The peptidyl-resin was subjected to the usual cleavage/deprotection mixture for 15 h, and a yellow colour was seen instantly on addition of the peptidyl-resin, due to the cleaved trityl cations in solution. A modified work-up procedure was then employed so as to avoid retritylation. Thus TFA, usually removed totally, was reduced in volume to about one sixth of the original. The resulting solution was then added to a large volume of water, to quench the trityl cations, before washing with *t*-butyl methyl ether. The crude product obtained was shown to contain 70% of free thiol groups. It was used in the following cyclisation reaction immediately without further purification, since we intended to minimise losses of material where possible. Simple air oxidation was the method selected, and no free thiol groups were detected after a period of 15 h. Initial purification by gel permeation chromatography indicated a minimum amount of polymeric material had been formed. Following preparative RPHPLC, the pure product **(7)** was obtained in 68% yield (from the crude disulphydryl compound). The overall yield was 28%.

This method has therefore resulted in a much improved yield of product. The advantage of the procedure is its relative simplicity, where

the cysteine protecting groups were cleaved very easily as part of the cleavage/deprotection process. The crude dihydro peptide **(48)** was subjected to oxidation as soon as it had been isolated and any purification delayed until cyclisation had been achieved. The alternative methods that were applied still have scope, once the work-up procedures have been optimised.

2.3.3. Cyclic MCH(5-17) (5)

Exocyclic, and ring subunit, analogues of MCH have been successfully synthesised. The importance of the exocyclic residues for binding/activity would be further tested by syntheses of analogues where either the C- or N-terminal residues are absent. Activity studies carried out by other workers[13a] on compounds of this nature have indicated that the C-terminal residues are more critical to MCH activity in teleosts compared to the N-terminal sequence. Therefore, the synthesis of cyclic MCH(5-17) **(5)** was attempted to test the effect on activity of the introduction of the C-terminal fragment. Thus the [Trt-$Cys^{5,14}$]-peptidyl-resin was required.



(5)

Solid phase synthesis proceeded in the usual manner. The first residue to be introduced was valine and this was achieved using the preformed anhydride double-couple procedure, with the usual amount of DMAP. The quantitative ninhydrin colour test indicated that a resin coverage of 77% had been attained and the synthesis was continued. All couplings were complete after 25 min except for Arg^9 (60 min) and Cys^5 (90 min).

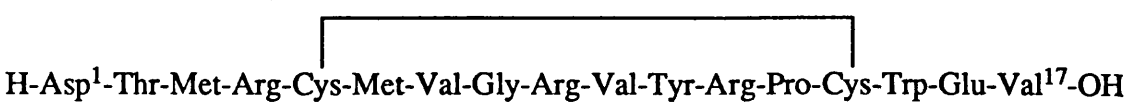
The peptidyl-resin was then subjected to a trial cyclisation adopting the same procedure as that found to be most successful in the synthesis of cyclic MCH(5-14) **(7)**. The optimum period for cleavage/deprotection was 7 h since the troublesome combination of arginine and tryptophan was again present. However, the crude linear dihydro peptide isolated was found to be fairly insoluble in the aqueous buffer used for the air oxidation process. Yields of the cyclic product subsequently isolated were very low

and a large amount of polymeric material was isolated. Therefore the procedure was modified slightly, where the crude disulphydryl compound was not actually isolated but air oxidation was allowed to proceed in the combined aqueous layers obtained from work-up of the cleavage/deprotection. The aqueous medium was adjusted to pH 7.6 by addition of ammonium hydroxide solution and was left to stand for 30 h. Following lyophilisation, the crude product was obtained in the presence of a large quantity of ammonium trifluoroacetate since the air oxidation solution was contaminated with TFA. Removal of the salt was easily accomplished by solid phase extraction using a small C_{18} silica cartridge, to yield the crude cyclic peptide. At low concentration of acetonitrile, the peptide remains bound to the column while the salt is not retained and removed by washing. The peptide can then be eluted at higher concentrations of acetonitrile.

The yield of product following gel permeation chromatography was 28% overall. Only a small portion was further purified by preparative RPHPLC since analytical RPHPLC indicated good purity of material following gel permeation chromatography. The modified air oxidation procedure minimised the risk of polymerisation occurring, since cyclisation was achieved before any lyophilisation process, during which premature and uncontrolled disulphide bond formation can occur.

2.3.4. MCH (1)

The synthesis of MCH **(1)** was the ultimate target in the first stage of the synthetic program. Bioassay of the product obtained would be a true test of integrity and of the synthetic methods so far employed.



(1)

The solid phase assembly was accomplished in parallel to that of cyclic MCH(5-17) **(5)** where the synthesis was continued until the complete MCH sequence had been assembled. Each remaining coupling was complete after 25 min.

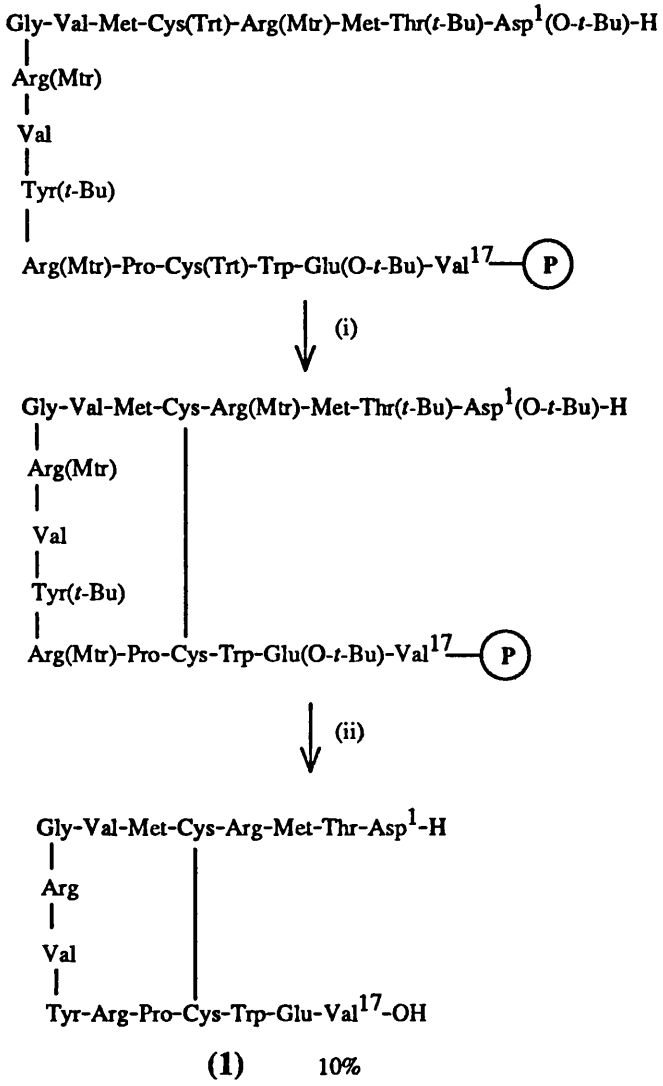
Following cleavage/deprotection (7 h optimum), the cyclisation procedure adopted was identical to that employed in the synthesis of cyclic MCH(5-17) (**5**) (see Section 2.3.3.), where a higher yield of product was obtained than if the dihydro peptide was isolated before air oxidation. The crude peptide was subjected to the usual vigorous purification procedures to afford the homogeneous product (**1**). The overall yield was only 13% but the crude product had not been as pure as would have been hoped.

A sample of synthetic MCH from another source (Peninsula Laboratories) was generously provided by Dr. Bridget Baker and shown to give rise to a peak corresponding in elution position exactly with that of our synthetic product. All spectral data indicated that the synthesis had been successful.

2.3.5. Synthesis of MCH (1) by Cyclisation on the Solid Support

Disulphide cyclisations performed on polystyrene and polyamide solid supports have been cited in the literature, and the reader is referred to some examples.[91] The utilisation of the pseudo-dilution properties of peptides linked to polymers, on the synthesis of disulphide bonds, should favour the desired intra-molecular reaction over the inter-molecular process. Thus, if the polymer-attached molecules are sufficiently distant from one another, then the intra-molecular cyclisation should predominate since it is rapid relative to the competing inter-site reaction. Solubility problems, which can lead to poor yields in solution, are also circumvented.

As part of our synthetic studies, an alternative preparation of MCH was attempted, by cyclisation on the solid support. The [Trt-$Cys^{5,14}$]-peptidyl-resin of MCH was used for this synthesis, as depicted in **Scheme 12.** In previous syntheses, Trt groups had been removed by TFA, however, in this case, undesired cleavage of peptide from the resin would also occur. Iodine was used to cause successive deprotection and disulphide bond formation. The medium of choice for the reaction was DMF. This solvent freely permeates and solvates the peptidyl-resin. Tryptophan was added as a scavenger to guard against iodination of this residue. To suppress oxidation of $Met^{3,6}$ the reaction was performed under nitrogen and methionine added. Iodination of tyrosine was not problematic since the phenolic hydroxyl was protected as the *t*-Bu



Scheme 12

ether. Following a 25 min reaction period, the removal of excess reactants was easily accomplished by various washing procedures. Cleavage/deprotection of the peptidyl-resin was achieved by treatment with a TFA-phenol mixture containing methionine and tryptophan as scavengers for these residues. It would also have been possible to use ethyl methyl sulphide in place of methionine. The usual TFA-EDT-phenol mixture could not have been used since EDT could cause reduction of the preformed disulphide bond. The characteristic yellow colour due to trityl cations in solution was not evident, indicating that they had been removed by the iodine treatment.

Cleavage of the peptide from the resin and removal of *t*-Bu protecting groups should be complete after 2 h. Therefore, the resin was removed at this stage to further prevent any back-addition of the detached peptide to the resin. The filtrate was left to stand for a further 5 h deprotection period before work-up, to isolate the crude peptide, which was satisfactorily purified to furnish a homogeneous product in 10% overall yield. The thiol test indicated the absence of free sulphydryls. The product was identical to that prepared by the previous methodology.

Thus it appears that disulphide bond formation has been achieved successfully by cyclisation on the solid support. Biological assay of the product will be a further test that MCH has been synthesised by this method. The crude material gave rise to a peptide peak on analytical RPHPLC that did not show broadening, as is characteristic of polymeric material. Purification, by preparative RPHPLC alone, was found to be satisfactory. In the previous synthesis of MCH the peptide peak of crude material was much less sharp though it improved dramatically on gel permeation chromatography using Sephadex LH20. All the indications are that the intra-molecular reaction has been favoured by cyclisation on the solid support.

Loss of peptide may have occurred during the cleavage/deprotection stage, due to irreversible attachment to the resin, leading to the disappointingly low yield. The Mtr protected/modified tryptophan peptide was also a contaminant.

With further optimisation, the advantages associated with the use of polymers as supports for cyclisation reactions may lead to greatly improved synthetic procedures, indeed this has been an area of much fruitful study.[92]

Homogeneous samples of each peptide, whose synthesis has been discussed, were submitted for bioassay. The analogues [DCys^{14}] cyclic MCH(5-14) (**44**) and [O-Met^{6}] cyclic MCH(5-14) (**47**) were also included out of interest, though they had not been intentionally prepared.

2.4. *STRUCTURE-ACTIVITY STUDIES

The structure-activity studies may provide an important insight into the receptor regulating pigment granule movements within melanophores.

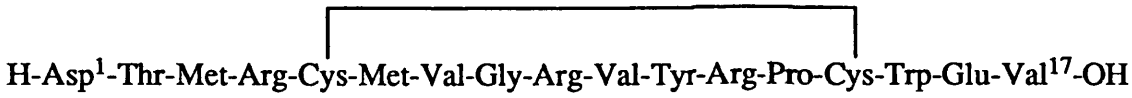
Rigorously purified samples of all peptides, whose synthesis has been considered thus far, were tested for agonist MCH bioactivity in the *in vitro* melanophore assay,[4] using scales of the Chinese grass carp *Ctenopharyngodon idellus*. Addition of MCH to the scales of teleost fish results in a lightening effect, due to melanosome aggregation within melanophores; such is the basis of this assay which has so far proved to be the most sensitive for MCH, though several have been utilised.[93] The EC_{50} (concentration required for half maximal pigment aggregation) was measured for each sample peptide. Thus the percentage potency relative to the reference synthetic salmonid MCH (100% activity) was calculated using the relationship:

$$\% \text{ potency} = \frac{EC_{50}\ \text{MCH}}{EC_{50}\ \text{sample}} \times 100$$

The % MCH activities are shown in **Table 4.** Parallelity of the dilution curves for each fragment with that of MCH itself is also indicated. These are effectively dose-response plots and an example is given in **Figure 20** for selected analogues. Dilution curves that were observed to be parallel to that of MCH may be an indication that the respective fragments interact similarly at the receptor site to the parent peptide.

Table 4

Melanin Concentrating Activity of Peptides *in vitro* Bioassay[a]



(1)

Fragment	% MCH Activity	Parallelity
linear MCH(15-17) **(17)**	2.1×10^{-4}	P
[Acm-Cys^{14}] linear MCH(14-17) **(18)**	1.0×10^{-4}	P
[Acm-Cys^{14}] linear MCH(13-17) **(19)**	2.0×10^{-4}	P
[Acm-Cys^{14}] linear MCH(12-17) **(20)**	2.2×10^{-4}	P
[Acm-Cys^{14}] linear MCH(11-17) **(21)**	2.0×10^{-4}	P
[Acm-Cys^{14}] linear MCH(10-17) **(22)**	2.7×10^{-3}	P
[Acm-Cys^{14}] linear MCH(9-17) **(23)**	5.8×10^{-3}	P
[Acm-Cys^{14}] linear MCH(8-17) **(24)**	5.0×10^{-3}	P
[Acm-Cys^{14}] linear MCH(7-17) **(25)**	4.3×10^{-3}	P
[Acm-Cys^{14}] linear MCH(6-17) **(26)**	9.0×10^{-3}	P
[Acm-Cys^{5,14}] linear MCH(5-17) **(27)**	1.7×10^{-2}	P
[Acm-Cys^{5,14}] linear MCH(4-17) **(28)**	2.0×10^{-1}	P
[Acm-Cys^{5,14}] linear MCH(3-17) **(29)**	3.2×10^{-1}	P
[Acm-Cys^{5,14}] linear MCH(2-17) **(30)**	5.2×10^{-1}	P
[Acm-Cys^{5,14}] linear MCH **(31)**	3.5×10^{-1}	P
[Acm-Cys^{5,14}, Phe^{11}] linear MCH **(32)**	1.0×10^{-1}	P
[Acm-Cys^{14}] linear MCH(9-14) **(33)**	ND	
[Acm-Cys^{14}] linear MCH(10-14) **(34)**	ND	
linear MCH(1-4,15-17) **(35)**	4.4×10^{-4}	P
linear MCH(1-4,Aha,15-17) **(36)**	3.8×10^{-4}	P
[Acm-Cys^{5,14}] linear MCH(5-14) **(43)**	1.7×10^{-4}	NP
[Acm-Cys^{5,14},Phe^{11}] linear MCH(5-14) **(45)**	7.0×10^{-5}	NP
[Acm-Cys^{5,14}] cyclic MCH(5-14) **(46)**	1.0×10^{-4}	P
cyclic MCH(5-14) **(7)**	1.5	P
[DCys^{14}] cyclic MCH(5-14) **(44)**	1.2	P
[O-Met^{6}] cyclic MCH(5-14) **(47)**	1.5	P
cyclic MCH(5-17) **(5)**	2.5	P
MCH **(1)**[b]	100	P
MCH (Peninsula Laboratories)	100	Ref

Aha = $-NH(CH_2)_6CO-$; ND = not detected; P = parallel; NP = non-parallel

[a]Each assay was performed at least twice, usually with periods of several months intervening, and procedures already described.[4] The % MCH activities quoted are therefore mean values.

[b]Refers to samples obtained by cyclisation both in solution and on the solid support.

Melanin Concentrating Activities of MCH-Like Peptides .

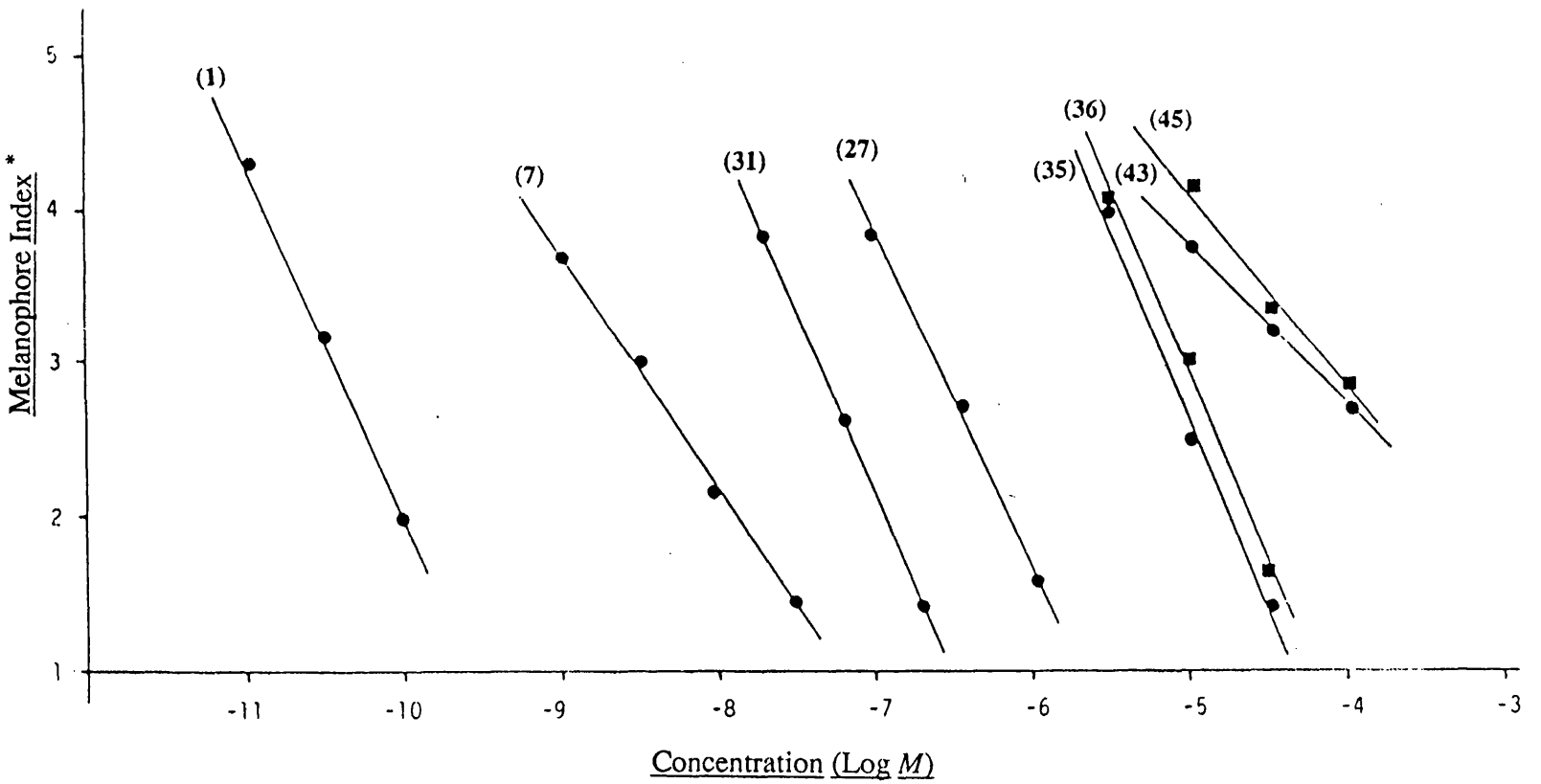


Figure 20

* In teleost melanophores , melanosomes are generally in the dispersed state , (5) .

MCH causes movement of melanosomes toward the cell centre , (1) .

2.4.1. Importance of Individual Amino Acids

The bioactivities of the sequential linear sequences **(17)**-**(31)** of MCH **(1)** will first be considered. It is evident from **Table 4** that there is a general trend of increasing potency as the MCH sequence is compiled from 2.1 x 10^{-4}% - 3.5 x 10^{-1}%, however, marked rises occur on the introduction of certain residues. From linear MCH(15-17) **(17)** to [Acm-Cys^{14}] linear MCH(11-17) **(21)** the MCH activity is fairly constant at *ca.* 2 x 10^{-4}%. However, on the introduction of Val^{10} an approximate 10-fold increase in activity occurs to 2.7 x 10^{-3}%. Further significant rises are seen on addition of Arg^{9} (5.8 x 10^{-3}%), Met^{6} (9.0 x 10^{-3}%) and Cys^{5}(Acm) (1.7 x 10^{-2}%) where activity roughly doubles each time. On introduction of the N-terminal exocyclic Arg^{4} residue the potency again shows a significant 10-fold increase to 2.0 x 10^{-1}%. An obvious conclusion to be drawn is that these amino acids may be particularly important for activity. The sequences studied were compiled from the C-terminus of MCH. It would indeed be of interest to study the relative activities of sequential fragments increasing in one amino acid at a time but from the N-terminus of MCH.

To test the importance of the Tyr^{11} residue, this amino acid was replaced by Phe in the linear MCH and 5-14 fragments. Unfortunately the activities of **(43)** and **(45)** were too low for any firm conclusions to be drawn about their relative potencies, and only a slight decrease in activity occurred for the Phe substituted linear MCH **(32)** compared to **(31)**.

During trial cyclisation reactions for the formation of the disulphide bond in the 5-14 subunit, we isolated [O-Met^{6}] cyclic MCH(5-14) **(47)**. Although the bioactivity study of the sequential fragments of MCH indicated the importance of Met^{6}, a comparison of the relative potencies of cyclic MCH(5-14) **(7)** and [O-Met^{6}] cyclic MCH(5-14) **(47)** shows that the oxidative modification of Met^{6} has had no effect. A similar finding resulted from studies by Baker et al[12] where the Met^{3}/Met^{6} sulphoxide analogues of MCH exerted virtually full bioactivity.

2.4.2. The Role of the N- and C-Terminal Sequences

Exocyclic fragments have been tested. These were the C-terminal tripeptide linear MCH(15-17) **(17)** and the sequences composed of the joined exocyclic sequences linear MCH(1-4,15-17) **(35)** and linear

MCH(1-4,Aha,15-17) **(36)**, in which the inserted Aha unit (-$NH(CH_2)_6CO$-) spaces the terminal sequences the same distance apart as in the native peptide.

Each of the three peptides elicited full intrinsic activity and although the potency was very low, the dose response plots appeared parallel to that of the reference parent peptide **(Table 4 and Figure 20)**, which may suggest a similar interaction with the receptor. The ring structure, cyclic MCH(5-14) **(7)**, had a markedly higher potency on carp melanophores, which was not affected by the addition to the incubation medium of the exocyclic residues assembly **(35)** at a concentration of 10^{-6} *M*. Therefore, although the terminal sequences possess some intrinsic activity, their main role is possibly to assist in the positioning/binding of the ring sequence, to achieve optimal interaction with the active site of the receptor molecule.

The importance of the exocyclic sequences was also indicated by the relative bioactivities of the linear fragments [Acm-$Cys^{5,14}$] linear MCH(5-14) **(43)**, [Acm-$Cys^{5,14}$] linear MCH(5-17) **(27)** and [Acm-$Cys^{5,14}$] linear MCH **(31)** which show a progressive increase from 1.7×10^{-4}% to 1.7×10^{-2}% to 3.5×10^{-1}%.

The same conclusions may also be drawn by a consideration of cyclic MCH(5-17) **(5)** and cyclic MCH(5-14) **(7)**. The analogue **(5)** possessing the C-terminal sequence is more potent than **(7)** where both the N- and C-terminal residues are absent. It would be interesting to test the activity of the cyclic MCH(1-14) **(6)** fragment in our assay to determine the relative importance of each exocyclic sequence. However, of the analogues prepared in our study, full bioactivity has so far only been elicited by the synthetic MCH **(1)**, prepared by cyclisation both in solution and on the solid support. Earlier bioactivity studies have been carried out by other workers on cyclic MCH(5-14) **(7)**, cyclic MCH(5-17) **(5)** and cyclic MCH(1-14) **(6)**. The results of these investigations, displayed in **Table 5**, are apparently at variance with one another, and with our results. In independent studies, full MCH bioactivity was found to be exerted either by (a) each analogue[8] or by (b) cyclic MCH(5-17) **(5)** alone,[13] where the C-terminal sequence was more important than the N-terminal residues. Each bioassay however used melanophores from different fish species and it is possible that the discrepant results reflect differences in the melanophore receptor requirements of the species in question.

Table 5

%Activities of MCH Fragment Analogues

	cyclic MCH(1-14)	cyclic MCH(5-17)	cyclic MCH(5-14)
	(6)	**(5)**	**(7)**
(a)	100	100	100
(b)	10	100	1.0

(a) Tested on scales from *Sarotherodon mossambicus*[8]

(b) Tested on skin from *Synbranchus marmoratus*[13]

It is of interest that [Acm-Cys^{5,14}] linear MCH(5-14) **(43)** and [Acm-Cys^{5,14},Phe^{11}] linear MCH(5-14) **(45)** were the only analogues tested whose dilution curves were, by visual inspection, clearly not parallel with that of MCH itself, suggesting that they do not interact normally with the receptor. The derivatives [Acm-Cys^{5,14}] linear MCH(5-17) **(27)** and [Acm-Cys^{5,14}] linear MCH **(31)**, however, do show parallel dilution plots, which again suggest the importance of the exocyclic residues in correctly positioning/binding the molecule to its receptor site.

2.4.3. The Requirement of the Cystine Bridge

The finding that [NO_2-Tyr^{11}]MCH and [I-Tyr^{11}]MCH both have reduced activity[8,12] suggests that the residues in the region of Tyr^{11} may be important. However, the fragments [Acm-Cys^{14}] linear MCH(9-14) **(33)** and [Acm-Cys^{14}] linear MCH(10-14) **(34)** showed no detectable activity when tested up to a concentration of 10^{-4} *M*, indicating that this sterically crowded site may require the restraint of the 5-14 cystine bridge to adopt the active conformation. The requirement of the cystine bond for activity

was therefore rationalised by a consideration of the relative potencies of [Acm-Cys^{5,14}] linear MCH(5-14) **(43)**, [Acm-Cys^{5,14}] cyclic MCH(5-14) **(46)** and cyclic MCH(5-14) **(7)**. These are the linear, Cys-Cys amide bond cyclised and Cys-Cys disulphide ring closed analogues of the subunit residues 5-14. Both the linear and amide bond cyclised fragments had very low % MCH activities of the order of 10^{-4}. In contrast, the cyclic cystine bridged fragment **(7)** was exceedingly more potent at 1.5%, indicating that the disulphide linkage is essential to maintain the biological activity, promoting the active conformation. The amide bond of the corresponding fragment [Acm-Cys^{5,14}] cyclic MCH(5-14) **(46)**, would profoundly alter the spatial arrangement of the adjacent amino acid residues. It is interesting to note that the diastereomeric form [DCys^{14}] cyclic MCH(5-14) **(44)** had a very similar activity to the unracemised product **(7)**; thus, change of chirality of Cys^{14} in the disulphide bond has had little affect.

2.4.4. Conclusions of the Structure-Activity Studies

Structural features thought to be essential for receptor binding and activity have arisen from the studies carried out so far.

It is evident that both the N- and C-terminal sequences are important for MCH bioactivity, and it is proposed that the exocyclic residues assist the positioning/binding of the molecule to achieve maximal receptor site interaction. In addition, the active conformation, probably a property of the 5-14 ring sequence, is promoted by the cystine bridge **(Table 6)**. This observation is in accord with those of other groups.[8,12] *

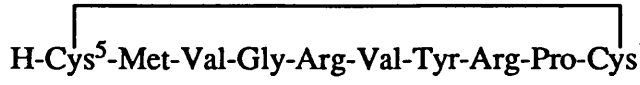
Table 6

Importance of Cyclic Conformation for MCH Bioactivity

Fragment	% Potency
H-Asp[1]-Thr-Met-Arg-Cys-Met-Val-Gly-Arg-Val-Tyr-Arg-Pro-Cys-Trp-Glu-Val[17]-OH **(1)**	100
H-Asp[1]-Thr-Met-Arg-Cys(Acm)-Met-Val-Gly-Arg-Val-Tyr-Arg-Pro-Cys(Acm)-Trp-Glu-Val[17]-OH **(31)**	$3{\cdot}5 \times 10^{-1}$
H-Cys[5]-Met-Val-Gly-Arg-Val-Tyr-Arg-Pro-Cys-Trp-Glu-Val[17]-OH **(5)**	2·5
H-Cys[5](Acm)-Met-Val-Gly-Arg-Val-Tyr-Arg-Pro-Cys(Acm)-Trp-Glu-Val[17]-OH **(27)**	$1{\cdot}7 \times 10^{-2}$
H-Cys[5]-Met-Val-Gly-Arg-Val-Tyr-Arg-Pro-Cys[14]-OH **(7)**	1·5
H-Cys[5](Acm)-Met-Val-Gly-Arg-Val-Tyr-Arg-Pro-Cys[14](Acm)-OH **(43)**	$1{\cdot}7 \times 10^{-4}$
Cys[5](Acm)-Met-Val-Gly-Arg-Val-Tyr-Arg-Pro-Cys[14](Acm) **(46)**	$1{\cdot}0 \times 10^{-4}$

2.5 *HIGH-FIELD ^{1}H N.M.R. STUDIES

On consideration of our results from the structure-activity studies, the cyclic MCH(5-14) **(7)** subunit was chosen for independent ^{1}H n.m.r.



(7)

analysis on the basis that it was one of the more potent analogues in the biological screen and in addition would give simpler spectra than the parent peptide. The linear analogue [Acm-Cys^{5,14}] linear MCH(5-14) **(43)** was also studied by ^{1}H n.m.r. as a comparison.

H-Cys^5(Acm)-Met-Val-Gly-Arg-Val-Tyr-Arg-Pro-Cys^{14}(Acm)-OH

(43)

It was our intention that primary structural confirmation of cyclic MCH(5-14) **(7)** would arise from the necessary, preliminary, high-field ^{1}H n.m.r. studies required to give information on preferred conformations in solution. Thus, ^{1}H resonance assignments for all peaks were required. To this end 1D, COSY, LRCOSY[94] and NOESY measurements were made. To locate higher structural features, a combination of NOESY and 1D difference NOE measurements were applied to locate short and long range through-space connectivities and enable some gross conformational characterisation to be made. In addition, variable temperature studies of the chemical shifts of backbone amide and arginine N$^\epsilon$H protons were performed to assess the extent of conformational homogeneity/heterogeneity.

Both cyclic MCH(5-14) **(7)** and [Acm-Cys^{5,14}] linear MCH(5-14) **(43)** were soluble in H_2O and DMSO. Spectra of each were measured in H_2O (containing not more than 20% D_2O), D_2O and DMSO. Very sharp signals were obtained in the first two solvents but broader spectra were observed in DMSO and in DMSO/$CDCl_3$ solutions (1:1 v/v).

2.5.1. Assignments

In our attempts to assign these spectra, attention was focused upon the characteristic proton resonances.

All residues, excluding the terminal Cys^5 and Pro^{13}, possess an exchangeable backbone amide NH proton, hence giving a total of eight for the peptide. In addition eleven backbone $C^{\alpha}H$'s were expected, where Gly^8 possesses two such protons. Each member is further characterised by the proton resonances of its side chain.

2.5.1.1. Assignments in H_2O and D_2O

The n.m.r. spectra of cyclic MCH(5-14) **(7)** and [Acm-$Cys^{5,14}$] linear MCH(5-14) **(43)** were obtained at 400 MHz in H_2O and D_2O at 25°C. For each compound identical spectra resulted from both their H_2O and D_2O solutions with respect to the non-exchangeable protons. In H_2O, all eight exchangeable amide protons and two $Arg^{9,12}$ imide protons were resolved. Therefore COSY measurements were carried out to identify each NH - $C^{\alpha}H$ pair and from these all eleven C^{α} protons were successfully located. These relationships established, the subsequent COSY measurements were made in a D_2O solution of cyclic MCH(5-14) **(7)**. The corresponding 1D and 2D spectra are shown in **Figure 21 a-c** and **Figure 22.** Assignments are displayed in **Table 7,** which were made with the aid of COSY **(Figure 22),** LRCOSY and NOESY. Assignments using similar measurements were made for [Acm-$Cys^{5,14}$] linear MCH(5-14) **(43)** in D_2O solution and are shown in **Table 8.**

400 Mz spectrum of cyclic MCH(5-14) (7) in D_2O

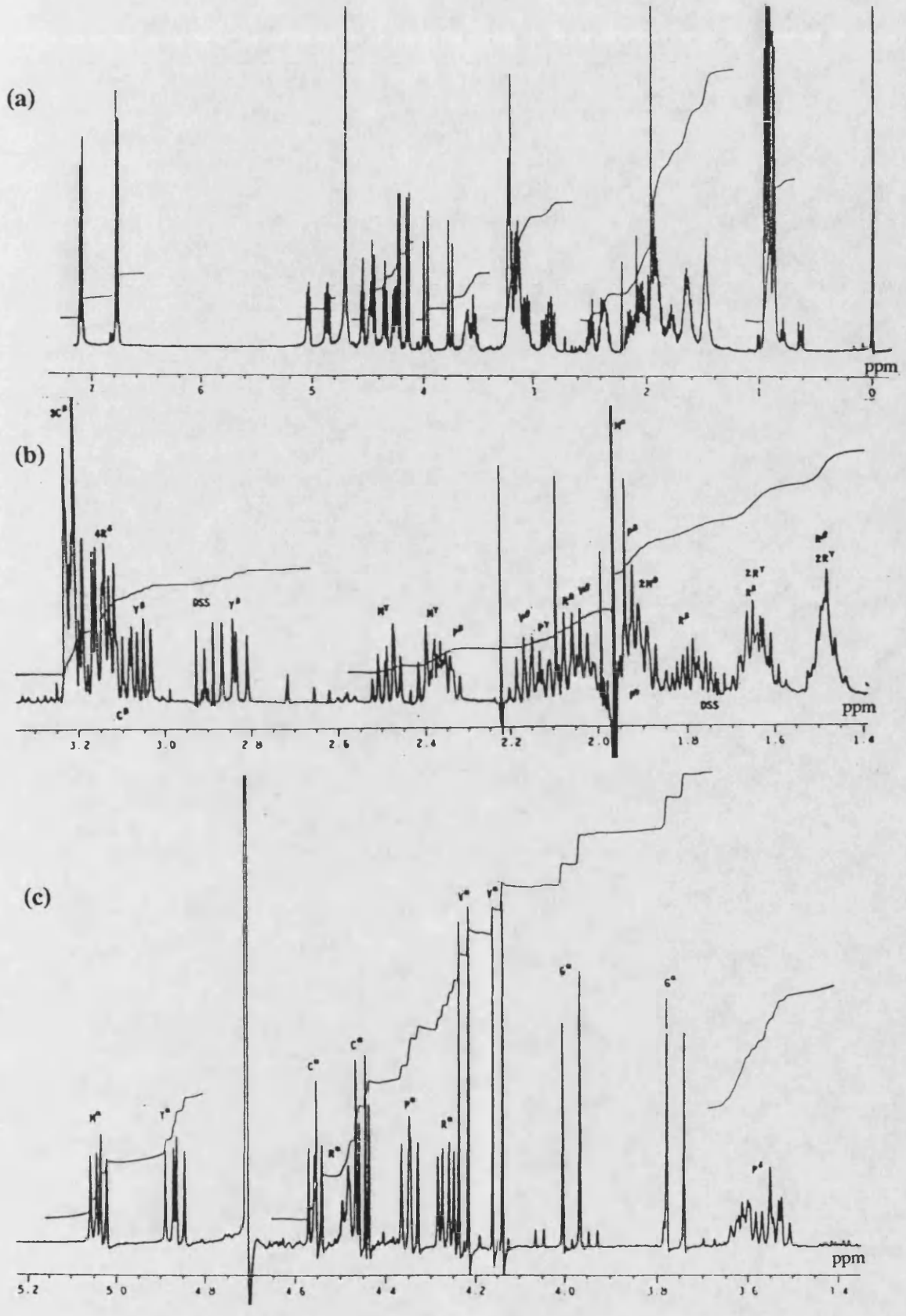
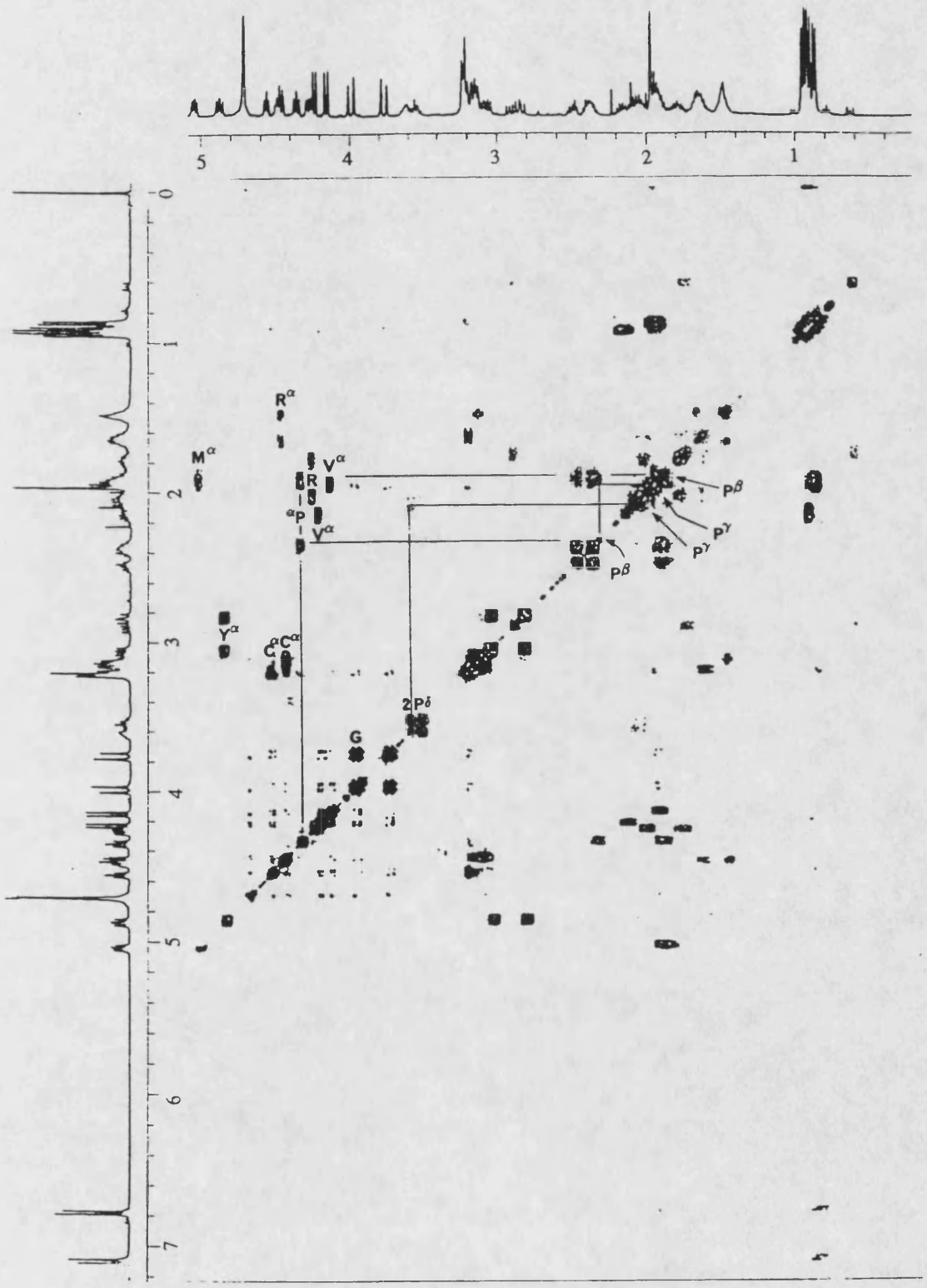


Figure 21



Cyclic MCH(5-14) (7) , D_2O , 25°C

Figure 22

Table 7

Chemical shift assignments for cyclic MCH(5-14) (7) in D_2O solution at 25°C

Residue		*NH	*J*	α	β	γ	δ	ϵ
5	Cys			4.55	3.24 3.10			
6	Met	8.84	7.9	5.04	1.95 1.91	2.39 2.49		1.97
7	Val	8.73	7.9	4.14	2.05	0.87 0.91		
8	Gly	8.90	5.7	3.98 3.76				
9	Arg**	7.88	7.6	4.47	1.48 1.65	1.48 1.7	3.13 3.18	7.18
10	Val	7.82	7.6	4.23	2.17	0.92		
11	Tyr	8.47	5.7	4.87	2.85 3.06		7.09	6.78
12	Arg**	8.72	7.6	4.27	1.79 2.08	1.48 1.70	3.13 3.18	~7.1
13	Pro			4.35	2.36 1.90	2.10 1.90	3.53 3.61	
14	Cys	8.63	6.6	4.45	3.23 3.21			

*Values obtained in H_2O at 25°C.
**Assignments interchangeable.

Table 8

Chemical shift assignments for [Acm-$Cys^{5,14}$] linear MCH(5-14) (43) in D_2O solution at 25°C

Residue		*NH	α	β	γ	δ	ϵ
5	Cys	8.10	4.58	3.20 3.02			
6	Met	8.55	4.41	2.30 2.02	2.58		
7	Val	8.15	4.08	2.01	0.89		
8	Gly	8.52	3.95				
9	Arg	8.15	4.56	1.62 1.77			7.14 or 7.16
10	Val	8.31	4.12	2.05	0.95		
11	Tyr	8.78	4.58	2.92 2.96			
12	Arg	8.11	4.36	1.58 1.74			7.14 or 7.16
13	Pro		4.63	2.02 2.08	2.29	3.51 3.61	
14	Cys		4.31	3.08 3.20			

*Values obtained in H_2O at 25°C.

Inspection of **Table 7** reveals that all the C^{β}, C^{γ}, $C^{\delta}$and C^{ϵ} protons have been assigned for each residue. The relative integrals in the 1D spectra showed agreement with the number of protons expected in each case. A comparison of the chemical shifts for cyclic MCH(5-14) (**7**) with the assignments made for the linear analogue, shows the greater dispersion of individual proton signals in the cyclic molecule. Of particular note is the differentiation of the Gly^8 C^{α} protons and the complete resolution of all the NH and $C^{\alpha}H$ protons for cyclic MCH(5-14) (**7**). This is consistent with the greater conformational restraints expected in the cyclic molecule. In addition, another difference of interest was the shift of the Met^6 $C^{\alpha}H$ from δ 4.41 ppm, for the linear analogue, to δ 5.04 ppm for the cyclic molecule, indicating possible deshielding of the Met^6 C^{α} proton in cyclic MCH(5-14) (**7**) compared to the linear peptide.

Hence assignments of proton resonances of cyclic MCH(5-14) (**7**) were made following the network of COSY cross-peaks for each residue. An example is depicted in **Figure 22** of the network of connectivities for the Pro^{13} residue, showing the coupling of the $C^{\alpha}H$ to $C^{\beta}H$'s, of $C^{\beta}H$'s to $C^{\gamma}H$'s and of $C^{\gamma}H$'s to $C^{\delta}H$'s, as indicated by the cross-peaks. All the $C^{\alpha}H$ assignments are also displayed. Certain resonances were instantly worth noting from the 1D spectrum that were readily assigned. These included the Gly^8 AX system as a doublet of doublets due to non-equivalence of the C^{α} protons of this residue. In addition the methyl resonances of the two $Val^{7,10}$ residues at δ 0.87-0.92 ppm and the methyl singlet of Met^6 at δ 1.97 ppm were also characteristic, as were the aromatic $C^{\delta}$and C^{ϵ} protons of the Tyr^{11} residue.

The sequencing of the individual members of the three pairs of like residues were made as follows:

(i) <u>Cysteines^{5,14}</u>

The cysteine resonances were identified by their networks of COSY connectivities and were clearly differentiated, since only one (Cys^{14}) possessed an amide NH. Thus, the COSY spectrum that was obtained in H_2O linked an NH at δ 8.63 ppm with a $C^{\alpha}H$-Cys at δ 4.45 ppm, therefore identifying this residue as Cys^{14} rather than Cys^5 **(Table 7).**

(ii) Valines [7,10]

The valine residues were recognised by their network of COSY cross-peaks. It was interesting to note that the characteristic methyl resonances appeared to exhibit non-equivalence for Val^7 (as two doublet of doublets, coupling to $C^{\beta}H$), but not for Val^{10} (only seen as a doublet). The two valines were distinguished by the one, strong, inter-residue cross-peak in the NOESY spectum run in H_2O, which linked the NH of Gly^8 at δ 8.90 ppm to the $C^{\alpha}H$ of Val at δ 4.14 ppm (**Table 7**), identifying this residue as Val^7. The chemical shift values of the non-exchangeable protons were unchanged between samples in D_2O and H_2O.

(iii) Arginines[9,12]

Using COSY, the network of connectivities for the protons of each arginine residue were established. However, no inter-residue connectivity was observed to allow assignment of the individual arginines to positions in the amino acid sequence, nor any NOE enhancements clear enough for this purpose.

One further correlation is worth noting. A LRCOSY measurement associating a $C^{\beta}H$ of the Tyr^{11} AMX system with its aromatic $C^{\delta}H$ confirmed the chemical shift assignment made to this residue.

2.5.1.2. Assignments in DMSO

The n.m.r. spectra were obtained at 400 MHz in DMSO at 25°C. Solutions of the two peptides (**7**) and (**43**) gave rise to broader signals and to a number of coincidences in δ values. Hence, only unambiguous or high probability connectivities established by NOE measurements were considered. The assignments of the protons of the cyclic analogue (**7**) in DMSO (**Table 9**) were based on a combination of COSY, NOESY and 1D difference NOE measurements. The $Val^{7,10}$ and $Cys^{5,14}$ pairs of residues were distinguished as before by the Val^7-$C^{\alpha}H$ to Gly^8-NH connectivity detected by NOESY measurements and the Cys^{14}-$C^{\alpha}H$ to Cys^{14}-NH cross-peak in the COSY spectrum. The $Arg^{9,12}$ residues were assigned with less certainty by inter-residue NOESY cross-peaks between the $C^{\alpha}H$ of Arg^9 and the NH of Val^{10}.

Table 9

Chemical shift assignments for cyclic MCH(5-14) (7)
in DMSO solution at 25°C

Residue		NH	α	β	γ	δ	ϵ
5	Cys		4.54	~3.0			
6	Met	*	4.52	1.90	2.96		
				2.09	2.56		
7	Val	7.73	4.12	1.90	0.84		
8	Gly	8.41	3.90				
			3.65				
9	Arg**	7.83	4.55	1.78	2.40	~3.1	9.23 or 8.70
				1.55			
10	Val	8.10	4.10	1.92	0.70		
11	Tyr	8.16	4.52	2.90			
				2.78			
12	Arg**	8.12	4.17	1.77	2.43	~3.2	9.23 or 8.70
				1.52			
13	Pro		4.33	2.02	2.40	~3.5	
14	Cys	8.08	3.98	2.92			
				3.24			

*Cross-peak absent.

**Assignment uncertain.

Having rigorously confirmed the primary structure of the cyclic MCH(5-14) (7) fragment, we turned our attention to the study of conformation.

2.5.2. Conformation

An initial conformational analysis was carried out by performing two separate studies. One such experiment was concerned with the determination of the temperature coefficients of the backbone amide and arginine $N^{\epsilon}H$ protons of cyclic MCH(5-14) (**7**). It was our intention to gain some understanding of the extent of conformational flexibility or constraint. In the second study 1D difference NOE studies were utilised, with the hope of indicating conformational features by locating long-range connectivities.

2.5.2.1. Temperature Coefficients Study

The variations with temperature of the chemical shifts of the backbone amide and Arg $N^{\epsilon}H$ protons of (**7**), in H_2O and in DMSO, are disclosed in **Tables 10** and **11** respectively. In H_2O, the shallowest slopes were shown by the Arg $N^{\epsilon}H$ protons, which is usually indicative of protection from solvent molecules. The remaining coefficients were large, indicating generally easy access by solvent molecules and little internal hydrogen bonding. However, in DMSO solution the arginine guanidinium NH's possessed the largest temperature coefficients, with uniformly low coefficients for the amide NH's; the noteworthy exceptions being the terminal Cys^{14} and Gly^{8} NH's. Changes with temperature of amide-proton chemical shifts of around 3 ppb/°C are regarded as being indicative of non-rigid peptide assemblies. In comparing the ratios cited in **Tables 10 and 11**, it is suggested that no major, rigid, hydrogen-bonded conformation exists in either of the solvents used. However, there appears to be a possibility of less molecular flexibility in DMSO solution. It is also interesting to note that the temperature coefficients of the NH amide protons of Gly^{8} are the largest of those in H_2O and are also relatively large compared to the other values in DMSO solution. This could be evidence for the flexibility of the Gly^{8} residue.

Table 10

Chemical shift of NH protons vs temperature (°C) for cyclic MCH(5-14) (7) in H_2O solution

Residue		20°	30°	40°	50°	60°	$\Delta\delta/\Delta T \times 10^3$
5	Cys						
6	Met	8.84	8.78	8.72	8.66	8.58	6.5
7	Val	7.83	7.78	7.72	7.68	7.64	4.8
8	Gly	8.90	8.78	8.67	8.56	8.46	11.0
9	Arg	7.88	7.83	7.78	7.74	7.69	4.8
10	Val	8.72	8.62	8.53	8.43	8.35	9.3
11	Tyr	8.47	8.37	8.29	8.20	8.13	8.5
12	Arg	8.72	8.65	8.58	8.49	8.41	7.8
13	Pro						
14	Cys	8.63	8.52	8.43	8.33	8.26	9.3
9	Argϵ*	7.18	7.15	7.12			3.0
12	Argϵ*	7.1		7.06	7.04	7.02	2.0

*Obscured by Tyr $C^{\delta}H$.

Table 11

Chemical shift of NH protons vs temperature (°C) for cyclic MCH(5-14) (7) in DMSO solution

Residue		20°	30°	40°	50°	$\Delta\delta/\Delta T \times 10^3$
5	Cys					
6	Met					
7	Val	7.70	7.68	7.64	7.62	2.7
8	Gly	8.41	8.38	8.30	8.28	4.3
9	Arg*	7.83	7.78	7.75	7.74	3.0
10	Val	8.10	8.12	8.07	8.05	3.3
11	Tyr	8.15	8.12	8.07	8.05	3.3
12	Arg*	8.12	7.78	7.75	7.73	3.3
13	Pro					
14	Cys	8.08	8.03	7.97	7.93	5.0
9	Argϵ*	9.21	9.15	9.10	9.06	5.0
12	Argϵ*	8.73	8.68	8.63	8.57	5.3

*Assignments not certain.

2.5.2.2. NOE Data

In D_2O solution, only intra-residue and a few inter-residue connectivities were observed. In contrariety, a number of longer range connectivities were detected in DMSO solution and are shown in **Table 12.** The most interesting of these involved the Tyr^{11} residue. Hence, by 1D Difference NOE, connectivities were indicated from Tyr^{11}-$C^{\epsilon}H$ to Pro^{13}-$C^{\alpha}H$ (1.5%), from Tyr^{11}-$C^{\delta}H$ to Val^{10}-$C^{\alpha}H$ (1%) and from a C^{β} proton of one of the Cys residues to Tyr^{11}-$C^{\epsilon}H$ (0.5%). These small but reproducible connectivities suggest an accessible conformation in which the Tyr^{11} aromatic ring occupies the centre-space of the peptide ring.

Table 12

Inter-residue NOE enhancements for cyclic MCH(5-14) (7) in DMSO solution

	Interacting atoms[a]		% enhancement	interatomic distances* Conformer 1	interatomic distances* Conformer 2
1	$C^{\delta}{}_{11}H$	$C^{\alpha}{}_{10}H$	1	6.6	3.9
2	$C^{\epsilon}{}_{11}H$	$C^{\alpha}{}_{13}H$	1.5	3.1	2.9
3	$C^{\epsilon}{}_{11}H$	$C^{\beta}{}_{5}$ or $C^{\beta}{}_{14}H$	0.5	4.1**	3.2**
4	N_8H	$C^{\gamma}{}_{7}H$	3	3.5	2.1
5	N_8H	$C^{\alpha}{}_{7}H$	15	2.1	2.4
5a	$C^{\alpha}{}_{7}H$	N_8H	20	2.1	2.4
6	$N_{10}H$	$C^{\gamma}{}_{9}H$	4	4.0	2.7
7	$N_{10}H$	$C^{\alpha}{}_{9}H$	6	2.1	2.3
8	$C^{\alpha}{}_{5}H$	$C^{\gamma}{}_{7}H$	2	6.0	5.6

[a]Subscripts refer to residue numbers.

* The interatomic distances (A°) are obtained from conformations observed in simulations, and where more than one hydrogen is designated represents the smallest value.

** N.m.r. assignments ambiguous; distances based on $C^{\beta}{}_{14}H$.

Such a conformation may require a transannular hydrogen bond for stabilisation. No signal which could have been assigned to the Tyr^{11}-OH proton was observed in either H_2O or DMSO. This may have been the result of rapid exchange with the terminal NH_2 and COOH protons (under the broad unresolved signal centred at δ 6.65 ppm in H_2O and δ 7.54 ppm in DMSO) or by severe broadening due to hydrogen bonding.

2.5.3. Conclusions from the ^1H N.M.R. Studies

The assignment of the various n.m.r spectra has confirmed the primary structure of the cyclic MCH(5-14) analogue (**7**). The most useful solvents of those employed were H_2O and D_2O. Moreover some interesting conformational features have arisen from our studies.

Temperature coefficients of backbone amide and $Arg^{9,12}$ $N^{\epsilon}H$ protons revealed:

(i) no indication of the existence of a particular constrained conformation, though less flexibility was inferred in DMSO solution,

(ii) possible flexibility of the Gly^8 residue.

NOE studies carried out in DMSO solution indicated:

(i) a number of long range connectivities,

(ii) the spatial position of the Tyr^{11} residue to be in the centre-zone of the ring portion, hence inferring that Tyr^{11} could be involved in a cross-ring hydrogen bond for stabilisation of such an arrangement.

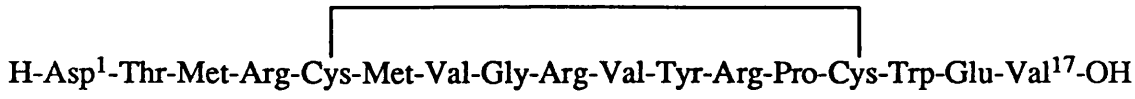
It is interesting to note that the Met^6-$C^{\alpha}H$ was notably deshielded in the cyclic analogue compared to the linear fragment. If the aromatic ring of Tyr^{11} occupied the centre-ring position in close proximity to Met^6 then such a deshielding effect could occur. This however must be treated tentatively, since a carbonyl group adjacent to the Met^6-$C^{\alpha}H$ in the cyclic analogue would also have this effect.*

2.6. *ANALYSIS OF CONFORMATION - A MOLECULAR DYNAMICS APPROACH

As the first stage is elucidating the shape and charge characteristics of MCH receptors, we have performed molecular dynamic simulations to search for accessible conformations of the hormone, in order to investigate the determinants for binding and activity. The results of these initial analyses could be used to suggest new analogues which will modify the conformational features of the peptide and further define the conformational requirements for activity.

Molecular dynamics methods have been employed recently to reveal accessible conformations and to characterise dynamic conformational transitions of proteins and peptide hormones.[95] In simulations the atoms follow a trajectory according to Newton's laws of motion with mobility determined by the temperature (or kinetic energy) of the system. Thus such a simulation could reveal a range of conformations which are accessible to the molecule at a chosen temperature, and when used in conjunction with energy minimisation, molecular dynamics becomes a powerful tool to study both the distinct conformational energy minima through which a system passes as well as the transitions between these low energy conformations.

In this initial study, the characteristic conformational features of the native peptide **(1)** were investigated, by applying molecular dynamics simulations and energy minimisation procedures. In addition, the importance of the disulphide bridge in determining the structural properties of the peptide and the effect of the terminal residues on the structure have been studied. Simulations were also carried out on two analogues, cyclic MCH(5-14) **(7)** and linear MCH(5-14) **(48)**. Both contain residues 5-14 of the ring portion, however in the cyclic fragment the disulphide bridge is present as in the native molecule whereas in the linear fragment cyclisation was removed resulting in loss of the cystine bond. These studies have been complemented by independent structure-activity (Section 2.4) and spectroscopic studies (Section 2.5). The comparisons and conclusions will be considered in subsequent sections.



(1)

(7)

H-Cys[5]-Met-Val-Gly-Arg-Val-Tyr-Arg-Pro-Cys[14]-OH

(48)

2.6.1. **Theory**

The molecular dynamics and energy minimisation techniques were applied using a valence force field software package enabling a detailed description of the energy surface of the molecule. In the valence force field[96] the potential energy of a molecular system is represented as an empirical function of the internal (valence) degrees of freedom and the interatomic distances. The analytical expression of the potential energy[57] includes, (a) strain energies which arise from deformations of internal coordinates like bond length, bond angle and torsion angle, and "cross terms" caused by coupling between deformations of two or more intervals, and (b) interaction energies which are a result of the exchange repulsion, dispersion and coulomb interactions between the non-bonded atoms. The parameters included in the various energy terms have been determined by fitting experimental crystal structure data, sublimation energies, molecular dipole moments, vibrational spectra and strain energies of small organic compounds. *Ab initio* molecular orbital calculations have also been used in conjunction with experimental data to give information on charge distribution, energy barriers and coupling terms. The effect of solvent has not been modelled.

Given the potential energy of the molecular system, the force exerted

on each atom by all other atoms in the system is defined and consequently the equations of motion are solved, yielding a detailed description of the dynamic behaviour of the system. The conformations accessed at different instances in the dynamics trajectory are then minimised and studied.

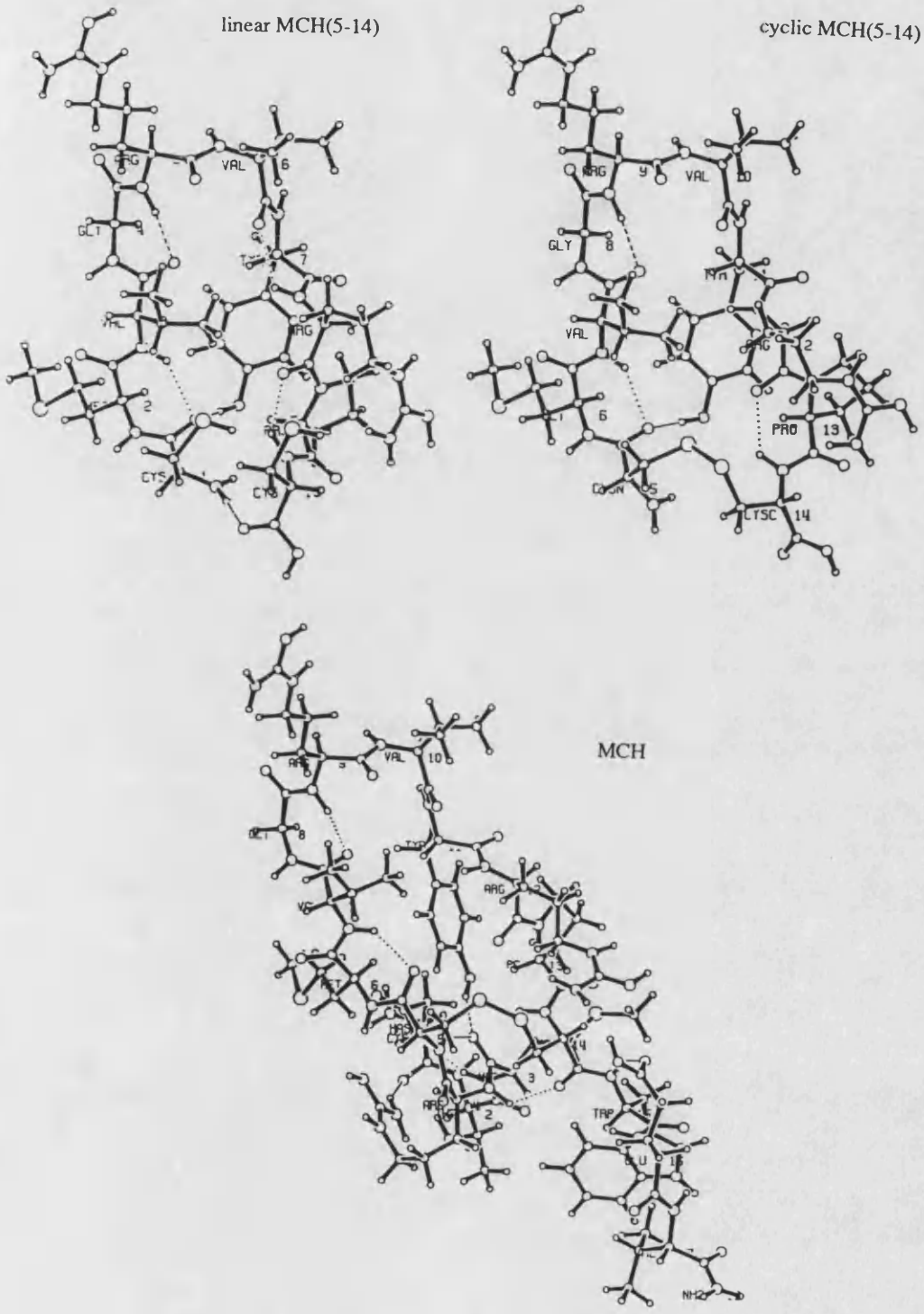
2.6.2. Method

The valence force field software package[57] was used to run the molecular dynamics simulations and energy minimisations. Extensive use was also made of the E and S PS300 picture system with the molecular graphics software package INSIGHT, to visually display and study the dynamics and minimum energy conformations.

The initial conformation of MCH (**1**) was built on the picture system. The residues 1-4 and 15-17 of the two exocyclic sequences were removed from this initial structure to generate the cyclic MCH(5-14) (**7**) fragment. The disulphide bond in this structure was then removed to generate the linear MCH(5-14) (**48**) structure. The initial backbone conformations of the two fragments were similar to the starting conformation of the full MCH (**1**) peptide.

To start the simulations, the initial conditions i.e. coordinates and velocities need to be specified. The three structures were minimised to relieve excessive strain and eliminate unrealistic motion before the molecular dynamics simulations were performed. These minimised conformations are shown in **Figure 23**. Initial random velocities consistent with a Maxwell-Boltzmann distribution for an average temperature of 300K were assigned to each of the atoms. Molecular dynamics simulations with a time step of 10^{-15} s, were performed for a total time period of 50 picosecond (50×10^{-12} s).

Chain torsion angles of energy-minimised conformations were analysed at 1 picosecond intervals along the dynamics trajectory of 50 picoseconds. To ensure convergence, each conformation was minimised and (ϕ,ψ) angles analysed for all amino acids.



Minimised conformations.

Figure 23

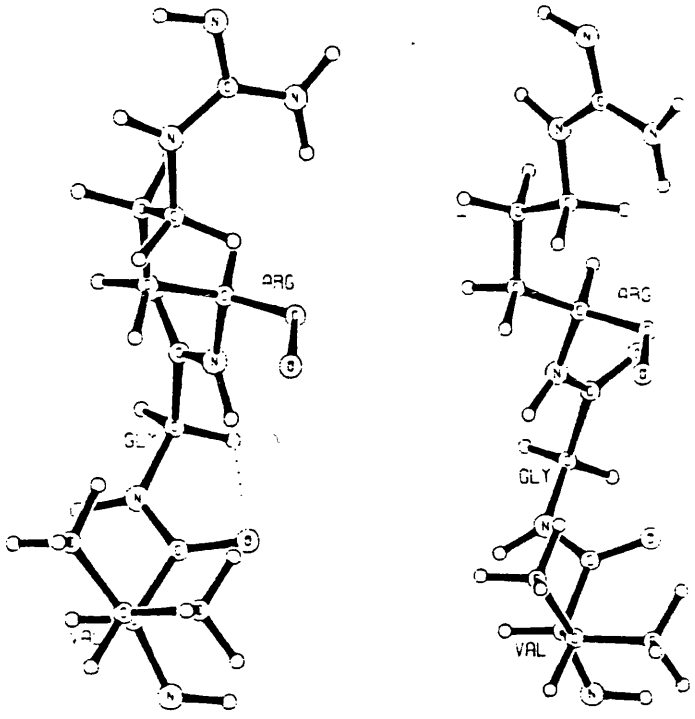
2.6.3. Backbone Conformations

In the first stage of the analysis of conformations accessed in the simulations, the general structure of the molecules, as determined by the conformation of the backbone, was focused upon. For each residue, (ϕ,ψ) values and the time periods during which they exist in the simulations were analysed. It is evident that MCH as well as the two analogues undergo a range of conformational changes. One of the most striking results revealed is the variability in conformational flexibility in different regions of the molecules.

The overall structural features of conformations accessed during these simulations are discussed in detail in the following sections in terms of backbone conformations, side-chain interactions and orientations of the disulphide bridge.

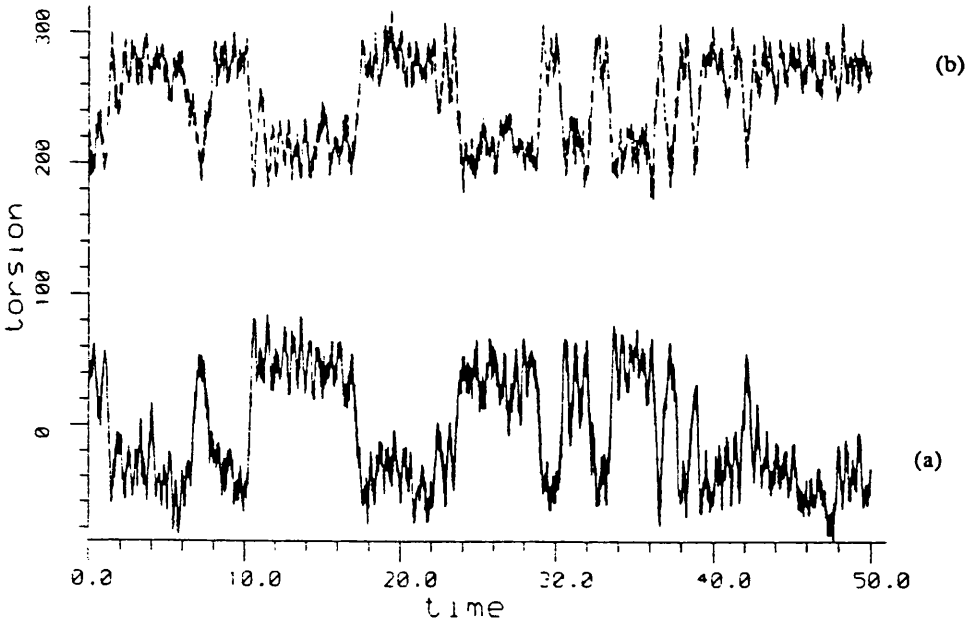
2.6.4. Regions of Conformational Flexibility

The major conformational flexibility of all three peptides is in the first half of the disulphide bridged loop (residues 5-14 of native MCH). Frequent and large conformational changes occur in the Gly^8-Arg^9 region of the peptide in all three systems. The Gly^8 residue exists mainly in either a γ-turn (-80,50) or an α-helical (-70,-40) type of conformation (**Figure 24**). The Arg^9 residue also occurs in two conformational states, which could be described as quasi-extended (-155,100) and a γ-turn (-80,100). The ϕ of Gly^8 and the ψ of Arg^9 show little change while conversely the ψ of Gly^8 and the ϕ of Arg^9 undergo concerted transitions. This tandem motion is illustrated in **Figure 25**, which shows the trajectories of ψ of Gly^8 and ϕ of Arg^9 in the native peptide over the 50 picosecond simulation period. Thus transitions between the conformational states persists throughout the simulations involving a simultaneous change in these torsion angles. In essence the transitions involve movement of the amide group connecting the two residues as a nearly rigid moiety, with the NH of Gly^8 and the CO of Arg hardly affected. This is also evident from **Figure 24** which shows the two alternative conformations in this region of the peptide.



γ-turn α-helical

Figure 24



The trajectories of (a) φ_{Gly} and (b) ϕ_{Arg} in the simulation .

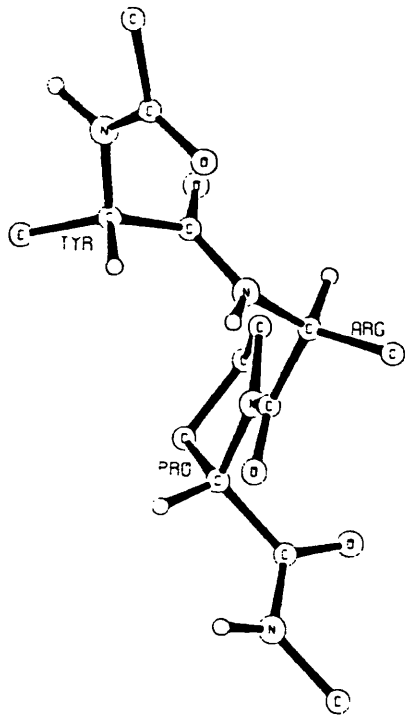
Figure 25

The other mobile residues in this region are Val^{7} and to some extent Met^{6}. They exist mainly in a γ-turn or a quasi-extended β-sheet structure.

Additional regions of structural flexibility are the exocyclic residues of native MCH. These regions are free to undergo conformational changes, since they are not constrained by cyclisation. However, the C-terminal residues (15-17) are much more mobile than the N-terminal residues (1-4).

2.6.5. Constrained Regions

The region from Val^{10} to Cys^{14} shows very little change. In particular the region from Tyr^{11} to Cys^{14} is in a stable helical arrangement (near the poly-proline helix) in all three peptides, as shown in **Figure 26.**



Constrained region of the peptide near Pro^{13}.

Figure 26

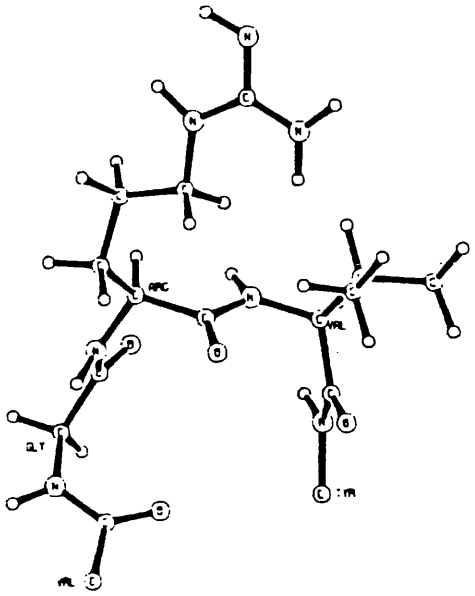
The rigidity of the Arg^{12}-Pro^{13}-Cys^{14} tripeptide can be explained in terms of conformational restrictions imposed by cyclisation and the presence of the proline residue. The disulphide bridge between Cys^5 and Cys^{14} imposes restriction on the ϕ of Cys. The pyrrolidine ring of the proline residue (with the side chain folded onto the backbone) imposes restrictions not only on the ϕ angle of proline but also on the conformations of the preceding amino acids.

Interestingly, although proline is known to be an initiator of β-turns, it does not participate in one in any of the peptides studied. Also, no β-turns were found in the proximity of Pro^{13}. A Tyr^{11}-Arg^{12} β-turn is ruled out as this also requires a hydrogen bond to the Pro^{13} amide nitrogen. In addition the cyclisation requirements of Cys^{14} prevent β-turns at Arg^{12}-Pro^{13} or Pro^{13}-Cys^{14}.

2.6.6. β-Turns

Given the cyclic nature of the peptides being studied, β-turns could be expected to play a major role in the conformational characteristics. Three "near" β-turns were located. Two of these, Gly^8-Arg^9 and Arg^9-Val^{10}, are in the cyclic segment of MCH and occur in all three peptides. The final one, involving Thr^2-Met^3 is found in the N-terminal sequence of MCH. The major difference between the (ϕ,ψ) angles characterising these turns and those expected for standard β-turns[97] is the value of the ψ_3 torsion angle. While the standard value of this angle in type I and II β-turns is near 0^o, here the values are around 80^0 and hence the associated 4→1 hydrogen bond is not found in any of the conformations.

Interestingly, the two β-turns in the cyclic region of the molecule occur simultaneously i.e. whenever a near type I Gly^8-Arg^9 β-turn occurs, the Arg^9-Val^{10} forms a near type II β-turn. This region of the peptide is shown in **Figure 27**. The β-turn in the N-terminal sequence of MCH is a near type I turn.



The region Gly^8-Arg^9-Val^{10}, showing the simultaneous occurrence of two β-turns.

Figure 27

2.6.7. Side-Chain Interactions

A regular feature in most of the conformations was a Tyr^{11}-OH to Cys^5-CO or Tyr^{11}-OH to Met^6-NH cross-ring hydrogen bond (see **Figure 23** for example). This was found in almost all the minimised conformations. The overall preference for this constraining feature was most significant. An additional simulation was carried out in which the Tyr^{11} side chain was moved away from the peptide ring. The cross-ring hydrogen bond was re-established in this additional simulation, even though it did not exist in the initial structure.

Another commonly found side chain-backbone hydrogen bond was between the Arg^{12} $N^{\epsilon}H$ and the C=O of Pro^{13}, especially in cyclic MCH(5-14) (**7**). Other noticeable features were the stretching out of the Met^6 side chain at regular intervals and interaction of the $N^{\epsilon}H$ of Arg^9 with

the NH of Val^{10}.

These interactions are likely to be modified by solvent when the intra-molecular processes noted above may be replaced by inter-molecular interactions with the solvent.

Another very important side-chain feature in cyclic peptides containing disulphide bridges is the orientation of the cystine bond as determined by the torsion around the S-S bond (θ). In the present studies, the value of θ in cyclic MCH(5-14) (**7**) and the parent peptide was ≈ +90° in the initial structures, and it remained near this value during the simulation, with no transitions to the other possible value, -90°. This is due to the high rotational barrier around the S-S bond (>10 kcal/mol) and consequently a very rare occurrence of transitions. However, this does not rule out existence of conformations with the other θ value. An exploration was carried out by removing the bridging atoms (C^{β} and S^{γ}) and inserting a new bridge with a conformation of θ = -90° in cyclic MCH(5-14) (**7**). It was found that the orientation of the cystine bridge had little effect on the overall conformation of the rest of the peptide.

2.6.8. Comparison of the Conformations of the Three Peptides

The overall backbone conformations of cyclic MCH(5-14) (**7**) and of the cyclic fragment of native MCH (**1**) are apparently similar. Our simulation reveals that the overall conformations and transitions of the linear MCH(5-14) (**48**) peptide fragment resemble those of the other two peptides. However, some conformational features were observed only for the linear fragment. In order to quantitatively evaluate the similarity in conformation between residues 5-14 of the full MCH system, cyclic MCH(5-14) and linear MCH(5-14), each of the conformations of one peptide was superimposed on each of the conformations of the other two. It was quite clear that the cyclic MCH(5-14) analogue is conformationally more closely related to the parent peptide than the linear analogue.

The comparison suggests a possible explanation for the lack of activity of the linear molecule. Although this analogue can adopt apparently similar conformations to those of the parent peptide, the deviation between them may be too large for effective binding and activity. In addition, the entropic factor arising from the need to select the binding conformation

from the many more available to the linear molecular will also contribute to its inactivity.

2.6.9. Conclusions

Molecular dynamics simulations and energy minimisation studies on the MCH systems have shown that there are two regions in the cyclic part of the peptide with very different conformational properties. These are the region around Gly^8 which shows mobility, and the region around Pro^{13} which is quite stable and rigid. The two conformations which the Gly^8 residue adopts suggests that two major families may be accessed. Moreover, a persistent feature was the Tyr^{11} phenolic cross-ring hydrogen bond. Thus, these analyses have led to a small set of key residues which are prime candidates for substitution, aimed at defining the conformational requirements for activity and consequently designing more active analogues. The similarity between MCH and cyclic MCH(5-14) conformations, suggests that the effect of the tail fragments on the ring conformation of MCH is marginal. The disulphide bridge appears to be of importance since conformations are adopted by the linear fragment which are significantly different. However, the orientation of the bridge is shown to have only a marginal effect on the overall conformation.

The results obtained so far have therefore established some conformational preferences for the hormone.*

2.7. CONFORMATIONAL ANALYSES - A COMPARISON OF MOLECULAR DYNAMICS STUDIES WITH N.M.R. RESULTS

Investigations of the conformations of MCH and its analogues have been performed by independent theoretical and experimental studies, using molecular dynamics and high-field ^1H n.m.r. analyses respectively. To a great extent the results of NOE experiments (see **Table 12**, Section 2.5), carried out on cyclic MCH(5-14) (**7**), are consistent with accessible conformations obtained from simulations of the cyclic MCH(5-14) molecule. Four backbone-side chain NOE's have been found:

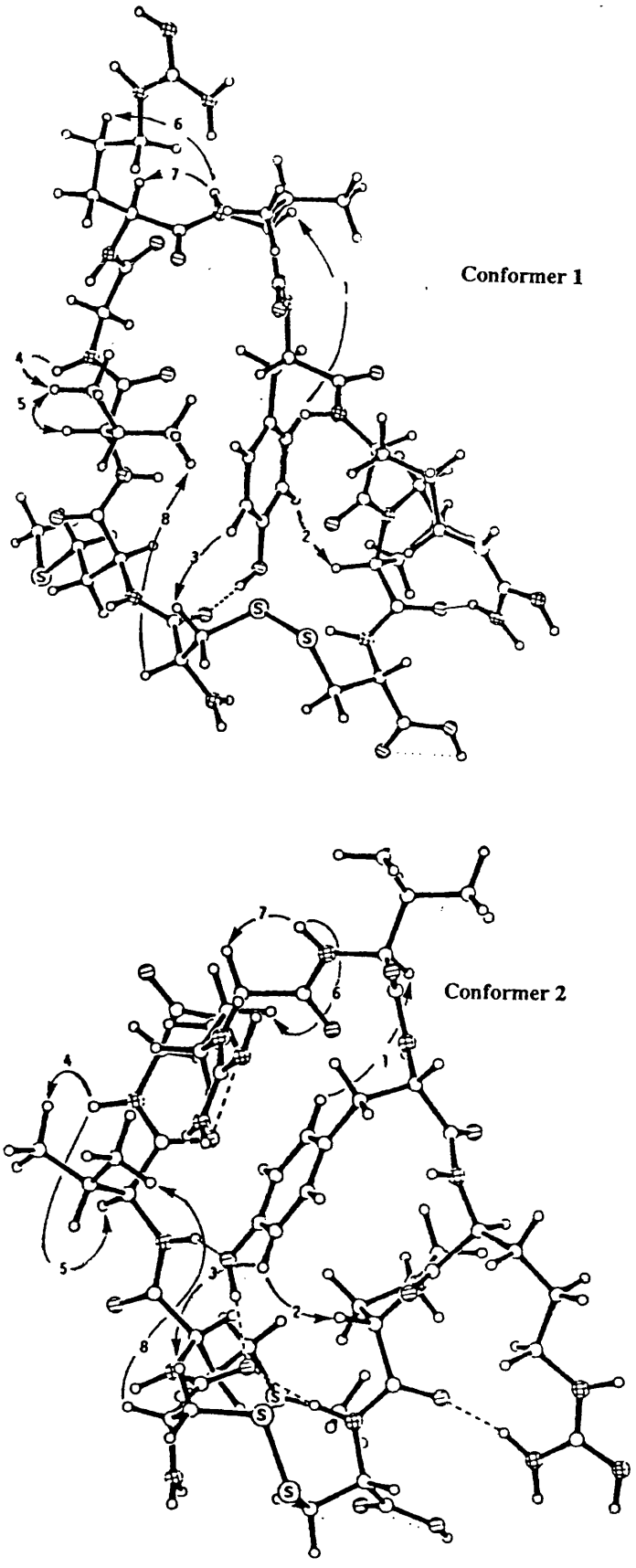
(a) Gly^8-NH to Val^7-$C^{\alpha}H$
(b) Val^{10}-NH to Arg^9-$C^{\alpha}H$
(c) Gly^8-NH to Val^7-$C^{\gamma}H$
(d) Val^{10}-NH to Arg^9-$C^{\gamma}H$

The first two NOE's, (a) and (b), are diagnostic[98] of a ψ value of 120° for Val^7 and Arg^9. This is in agreement with values obtained from the modelling studies. The connectivities indicated by (c) and (d) above are also consistent with the minimised conformations accessed by the dynamics simulations. In addition, of the four side chain-side chain NOE's, two are in accordance with most of the cyclic MCH(5-14) minima:

(i) Tyr^{11}-$C^{\epsilon}H$ to Cys^5-$C^{\beta}H$
(ii) Tyr^{11}-$C^{\epsilon}H$ to Pro^{13}-$C^{\alpha}H$

These connectivities, (i) and (ii), were of particular interest since they indicated the presence of a significant population of conformations in which the Tyr^{11} side chain occupies the centre space above the peptide ring.

All the NOE enhancements obtained on cyclic MCH(5-14) (**7**) in DMSO solution (given in **Table 12**, Section 2.5) are for convenience indicated on the conformers, 1 and 2, shown in **Figure 28**. These conformers are typical representations of the two families of accessible minimised conformations generated from the molecular dynamics simulation, characterised by the α-helical (conformer 1) or γ-turn (conformer 2) conformation of Gly^8. All minimised conformations from each family featured the transannular interaction involving the Tyr^{11} residue. The NOE enhancements observed are largely consistent with the simulated spatial arrangements. The great majority of these minimised conformations satisfied the spatial requirements for either six of the eight or seven of the eight observed NOE



Cyclic MCH(5-14), showing NOE enhancements.

Figure 28

effects, depending upon the family. In these, the Tyr^{11} phenolic OH hydrogen is close to the Cys^5 and to the Met^6 carbonyl oxygens. This could provide the hydrogen bond which may be necessary to stabilise such an arrangement. The existence of this feature is therefore suggested by the independent n.m.r. analyses and molecular dynamics simulations.

Apart from consistences of measured NOE enhancements with the simulated conformations, the region of conformational flexibility around Gly^8 has also been determined by molecular dynamics and separately by the n.m.r. studies of temperature coefficients.

It should be emphasised that no explicit constraints were incorporated in the molecular dynamics simulations. Considering the limited number of observed NOE enhancements, and consequently the small number of proximity constraints revealed by the experiments (relative to all possible proton pairs in the peptide), it is noteworthy that the simulations produced conformations which are compatible to such a degree with the n.m.r. experiments. Thus, this agreement lends credibility to the results of the simulations and indicates that important accessible conformations have been successfully generated.

It is important to mention recent findings of other workers[15b] which add further credence to our results. In attempts to determine the conformational structure requirements for MCH at its receptor, analyses have been carried out by applying ^1H n.m.r. assignments and conformational studies of MCH in water using NOE constraints and molecular modelling algorithms. A total of 69 NOE's were established in 90% H_2O-10% D_2O.

In agreement with our studies, molecular dynamics indicated a Gly^8-Arg^9 type I β-turn. In addition, a transannular NOE was observed between the Tyr^{11} aromatic protons to the $C^{\alpha}H$ of Met^6 and the NH of Val^7. There appeared to be no convergence upon a common structure, suggesting some flexibility in other parts of the molecule.

2.8. CONCLUSION OF THE STRUCTURE-CONFORMATION-ACTIVITY STUDY - "STAGE 1"

The preliminary, independent, experimental and theroretical studies have located salient conformational features and potentially active sub-structural units of the hormone. Moreover, conclusions reached from the separate structure-activity, high-field n.m.r. and molecular dynamics analyses have shown a marked compatibility.

The results of the "Stage 1" study are summarised as follows:

(1) Structure-activity studies suggest that the active conformation is probably a property of the 5-14 sequence and is promoted by the cystine bridge. The importance of the disulphide bond was also indicated by molecular dynamics simulations, where conformations were adopted by the linear MCH(5-14) **(48)** fragment which were markedly different to those of the native peptide.

(2) The importance of the N- and C-terminal sequences was shown by the structure-activity studies. It is suggested that they may assist in molecule binding, to elicit maximal receptor site interaction. Analysis of the bioactivities of the sequential linear sequences of MCH **(17)** - **(31)**, showed a marked increase in activity on introduction of the first of the N-terminal residues, namely Arg^4. Molecular dynamics simulations indicated that the exocyclic sequences do not influence the conformation adopted since their effect on the cyclic ring portion is marginal.

(3) Regions of conformation flexibility have been defined, together with constrained regions. The flexibility of the Gly^8 residue has been indicated by both molecular dynamics and n.m.r. analyses. The simulations revealed that Gly^8 adopts either a γ-turn or an α-helical conformation. Thus it is suggested that two major families of conformations may exist.

The molecular dynamics simulation indicated a particularly strained region around Pro^{13}. In addition, the Val^{10} residue showed little mobility and induced an increase in activity on its introduction in the structure-activity study of the linear sequential MCH fragments.

(4) The existence of β-turns and key residues involved in conformational determination has been predicted by molecular dynamics studies.

(5) There was remarkable agreement between the experimental NOE constraints determined by n.m.r. analyses and the accessible conformations obtained from the simulations. A dominant transannular tyrosine phenolic hydrogen bond has emerged from different dynamics starting points. Independent n.m.r. analyses have provided possible experimental validation of this feature. These findings strongly support the suggested importance of the Tyr^{11} side chain in stabilising the biologically active conformation.

The results obtained thus far have established a fundamental knowledge of the hormone and given some indications regarding the direction of further experimental and theoretical studies, as will be considered in the following Chapter.

Chapter 3

SYNTHESIS, BIOLOGICAL ACTIVITY AND CONFORMATIONAL STUDIES

"STAGE 2"

The major sub-structural conformational features defined by the "Stage 1" studies, have enabled certain amino acid substitutions to be predicted, which should result in definable conformational constraints. Thus, structure-activity and conformational studies can then be performed on the resulting fragments to further investigate these features.

The Tyr^{11} residue has been indicated to play a key role in determining the conformation of MCH. A dominant transannular phenolic hydrogen bond has emerged from both molecular dynamics and high-field n.m.r. studies. A simple substitution of Tyr^{11} by Phe enables further investigation of the importance of the Tyr^{11} side-chain conformation in determining the activity of MCH.

The importance of Tyr^{11} for activity was suggested by other workers,[8,12] and this prompted our earlier syntheses of [Acm-$Cys^{5,14}$,Phe^{11}] linear MCH **(32)** and [Acm-$Cys^{5,14}$,Phe^{11}] linear MCH(5-14) **(45)**. Unfortunately, no meaningful conclusions could be formed from comparisons of potencies with those of the corresponding Tyr^{11} peptides. The significance of Tyr^{11} for activity, may be tested more effectively, by a comparison of the relative potencies of disulphide cyclised analogues. To this end, [Phe^{11}] cyclic MCH(5-14) **(49)** was targeted, being the phenylalanine substituted analogue of cyclic MCH(5-14) **(7)**.

H-Cys^{5}-Met-Val-Gly-Arg-Val-Phe-Arg-Pro-Cys^{14}-OH

(49)

H-Cys^{5}-Met-Val-Gly-Arg-Val-Tyr-Arg-Pro-Cys^{14}-OH

(7)

In the regions of conformational flexibility, the purpose of the substitutions will be to reduce the conformational space which is most important for activity. Hence, Gly^8 is an obvious candidate for substitution. As is well known, the glycine residue has considerable freedom owing to the absence of a constraining group in its side chain. This is well reflected in the dynamics simulations, where the Gly^8 residue adopts γ-turn, and α-helical conformations, alternately. It is of interest, that the two main conformations adopted by Gly^8, are both favourable for L-residues. Substitution of Gly^8 by an α,α-dialkylated residue, or a D-residue, may show preference for one of these conformational types over the other, and throw more light on the biologically active conformation of MCH. To this end, the synthesis of the analogue [$DAla^8$] cyclic MCH(5-14) **(50)** was required, where Gly^8 is substituted by DAla. The corresponding Phe analogue, [$DAla^8$,Phe^{11}] cyclic MCH(5-14) **(51)** was also targeted, so that a comparison of the relative potencies of **(50)** and **(51)** may provide further verification of the importance of the Tyr^{11} residue.

H-Cys[5]-Met-Val-DAla-Arg-Val-Tyr-Arg-Pro-Cys[14]-OH

(50)

H-Cys[5]-Met-Val-DAla-Arg-Val-Phe-Arg-Pro-Cys[14]-OH

(51)

3.1. SYNTHESES OF CYCLIC ANALOGUES OF MCH WITH AMINO ACID SUBSTITUTIONS

3.1.1. [Phe^{11}] cyclic MCH(5-14) (49)

The synthesis was accomplished by treatment of [Acm-$Cys^{5,14}$,Phe^{11}] linear MCH(5-14) **(45)** with iodine, to effect the cyclisation. A similar procedure was used, as in the synthesis of cyclic MCH(5-14) **(7)**, with some alterations. Both a larger quantity of iodine and a much longer reaction time were required. The synthesis of **(7)** posed problems with removal of iodine, but it has since been found that treatment with zinc dust is extremely effective,[99] which can be removed by simple filtration. The pure peptide **(49)** was obtained in 14% yield from **(45)**.

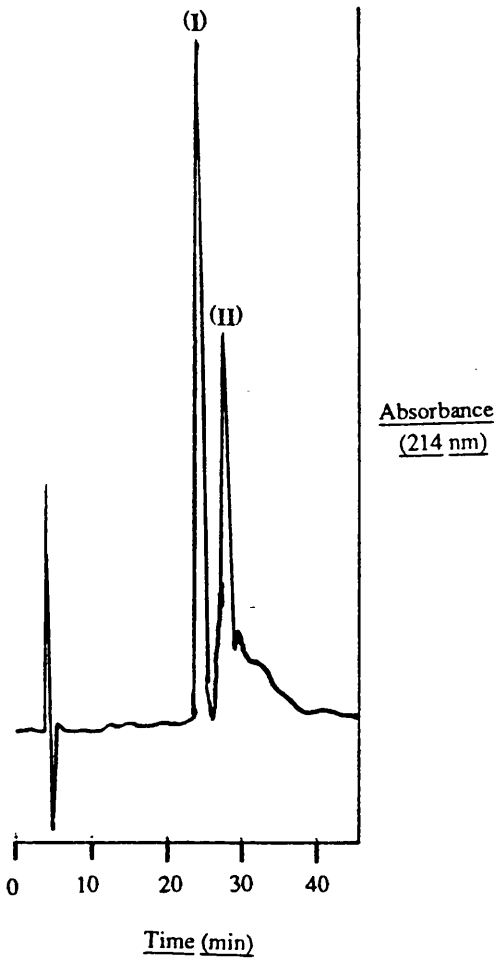
3.1.2. [DAla^8] cyclic MCH(5-14) (50)

Synthesis of [DAla^8] cyclic MCH(5-14) (**50**) was accomplished in a similar manner to the preparation of cyclic MCH(5-14) (**7**), where Trt groups were employed for the protection of Cys^{5,14} (see Section 2.3.2.3.). The first residue was introduced by three consecutive batches of Fmoc-Cys(Trt)-OPfp, with the reduced amount of DMAP. The quantitative Fmoc determination was carried out and indicated that 73% resin esterification had occurred. The synthesis was then continued, coupling the Fmoc-DAla residue as the preformed symmetrical anhydride. All couplings were complete after 25 min, apart from Val^7 (45 min). Following cleavage/deprotection of the peptidyl-resin and subsequent cyclisation by air oxidation, the crude peptide was subjected to gel permeation chromatography on Sephadex LH20. A product of excellent purity was obtained in 19% overall yield.

3.1.3. [DAla^8,Phe^{11}] cyclic MCH(5-14) (51)

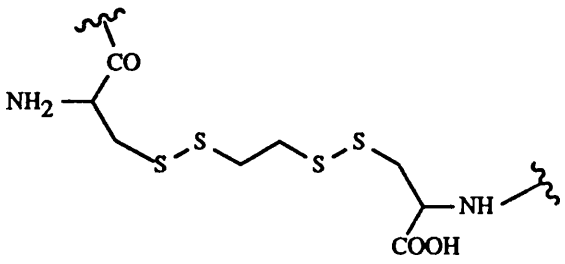
In a parallel synthesis to that of (**50**), the Phe analogue was prepared, substituting for Tyr^{11} at the appropriate point. An identical cyclisation and purification procedure was employed, to afford the desired compound in 44% overall yield.

In attempts to increase yields, the alternative aeration process was applied, where the aqueous solution from extraction, following cleavage/deprotection, was left to air oxidise, as in the synthesis of cyclic MCH(5-17) (**5**) (see Section 2.3.3.). However, a by-product (II) was formed during the aeration process, that was more retained on RPHPLC than the required product (I) (**Figure 29**). The contaminant was isolated by preparative RPHPLC, and analysis by F.A.B. mass spectrometry, revealed a peak at m/z 1272 $(M+H)^+$, compared to m/z 1180 $(M+H)^+$ for the desired product. It is proposed that the secondary product is the desired peptide, with the usual disulphide bridge interspersed by an ethanedithiol spacer (**52**). This would account for the observed mass difference. Formation of the by-product has most likely occurred during the aeration process, since residual amounts of EDT would be present in solution. No such by-product was isolated in the syntheses of cyclic MCH(5-17) (**5**), or MCH (**1**), where the same oxidation procedure was employed.



Synthesis of (51) : analytical RPHPLC trace of the air oxidation reaction showing the desired product peak (I) and the by-product peak (II) .

Figure 29



(52)

It was decided to treat the by-product with sodium borohydride in neutral buffered solution. If the EDT unit had been incorporated, then reduction should occur to afford the free dihydro form of **(51)**, which would then be allowed to undergo air oxidation to render the desired product. Indeed, analysis of this reaction by RPHPLC, revealed the by-product to be

no longer present, only the desired product (I) being evident. This added confirmation to our suggestion that the by-product had resulted from incorporation of EDT into the desired peptide.

It can therefore be concluded, that both DAla analogues, **(50)** and **(51)**, have been successfully prepared. A much higher yield of the Phe analogue was obtained, since less polymeric material appeared to have formed during the air oxidation process. The incorporation of EDT into the peptide was an unexpected yet interesting phenomenon.

3.2. *STRUCTURE-ACTIVITY STUDIES

The analogues **(49)**, **(50)** and **(51)**, were subjected to the *in vitro* melanophore assay,[4] in the same manner as has been previously applied (see Section 2.4.). The % MCH activities are shown in **Table 13**. The potency of the corresponding unsubstituted compound, cyclic MCH(5-14) **(7)**, is also indicated as a comparison.

3.2.1. Effect of the Substitution of Tyr^{11} by Phe

It is evident from **Table 13**, that substitution of Tyr^{11} by Phe, in the cyclic MCH(5-14) fragment, has resulted in a marked decrease in activity from 1.5% to 1.9×10^{-2}%. This reduction in potency is clear evidence for the importance of the Tyr^{11} side chain functionality in determining the biologically active conformation. This is shown from the relative potencies of [$DAla^8$] cyclic MCH(5-14) **(50)**, and [$DAla^8$,Phe^{11}] cyclic MCH(5-14) **(51)**, where the Phe analogue is less active.

3.2.2. Effect of the Substitution of Gly^8 by DAla

The replacement of Gly^8 by DAla in the cyclic MCH(5-14) fragment has effected a significant reduction in activity from 1.5% to 8.0×10^{-3}%. This substitution has given rise to a similar decrease in activity for the corresponding Phe analogues **(49)** and **(51)**.

It therefore appears that the biologically active conformation has been disrupted by this substitution. Moreover, the $DAla^8$ residue may have elicited a preference for one of the conformations (either γ-turn or α-helical) previously indicated to be equally possible for the Gly^8 residue.

Table 13

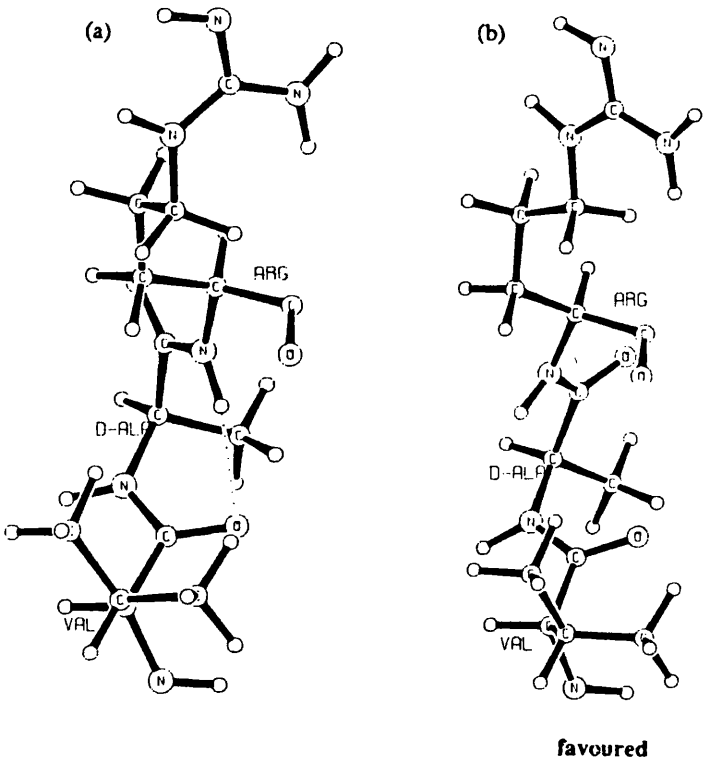
Melanin Concentrating Hormone *in vitro* Bioassay

Fragment		% MCH Activity	Parallelity
H-Cys^{5}-Met-Val-Gly-Arg-Val-Tyr-Arg-Pro-Cys^{14}-OH	**(7)**	1·5	P
H-Cys^{5}-Met-Val-Gly-Arg-Val-Phe-Arg-Pro-Cys^{14}-OH	**(49)**	$1{\cdot}9 \times 10^{-2}$	P
H-Cys^{5}-Met-Val-DAla-Arg-Val-Tyr-Arg-Pro-Cys^{14}-OH	**(50)**	$8{\cdot}0 \times 10^{-3}$	P
H-Cys^{5}-Met-Val-DAla-Arg-Val-Phe-Arg-Pro-Cys^{14}-OH	**(51)**	$4{\cdot}5 \times 10^{-4}$	P

If this is indeed true, then the biologically active conformation is unlikely to be that inferred by the DAla substitution.
A molecular dynamics analysis is therefore required to determine the effect of the modification on the conformational flexibility.

3.3. MOLECULAR DYNAMICS SIMULATION

The effect of the substitution of Tyr^{11} by Phe, in the cyclic MCH(5-14) fragment, has yet to be analysed by molecular dynamics. However, the replacement of Gly^8 by DAla in this analogue has been simulated, for each of the geometries (γ-turn and α-helical) that Gly^8 has been shown to adopt. The resultant plots are shown in **Figure 30.** It is initially evident that an α,α-dialkylated residue, or a D-residue, is required to enforce a preference in the regional conformation of the cyclic fragment, since for an L-residue the side chain would be displaced away from the backbone. Further examination of **Figure 30,** reveals that the α-helical conformation is favoured. The alternative γ-turn geometry is now much less likely, due to the hindrance of the DAla methyl group on the backbone atoms.*



Gly^8 substituted by DAla in , (a) γ-turn , and (b) α-helical conformations .

Figure 30

3.4. CONCLUSION OF THE STRUCTURE-CONFORMATION-ACTIVITY STUDY - "STAGE 2"

The structure-activity study has indicated that Gly^8 and Tyr^{11} are key residues in determining the biologically active conformation, since their substitution has resulted in substantial decrease in activity.

The substitution of Tyr^{11} by Phe, in the cyclic MCH(5-14) fragment, negates the possibility of the transannular hydrogen bond; a feature that persisted throughout the dynamics simulations and which was also indicated by the n.m.r. analyses. The lower activities of the Phe analogues adds further support to this proposed feature, and experimental and theoretical conformational analyses of these fragments are now required.

The molecular dynamics simulations suggested that substitution of Gly^8 by DAla in the cyclic ring portion of MCH is likely to favour an α-helical rather than a γ-turn conformation in this region of the peptide, thus reducing flexibility. The reduced potency of the $DAla^8$ analogues suggests that the biologically active conformation may require the adoption of the γ-turn geometry around Gly^8. The molecular dynamics simulation carried out previously (see Section 2.6.4.), indicated the possibility of a "near" type I Gly^8-Arg^9 β-turn. Support for this feature is provided by the recent revelations of other workers.[15b] However, the β-turn is only consistent with the α-helical conformation of Gly^8, which is contra-indicated by the observed activities. However, conformational studies of MCH ring-contracted analogues,[14] possessing MSH-like activity, revealed a preference for the α-helical geometry of Gly^8. It is possible that the α-helical conformation is more critical to MSH-like activity, and the γ-turn to MCH activity.

High-field ^1H n.m.r. analyses of [$DAla^8$] cyclic MCH(5-14) **(50)** and [$DAla^8$,Phe^{11}] cyclic MCH(5-14) **(51)** are currently in progress. It is our intention that such studies may indicate that changes in conformation have occurred on substitution of Gly^8 by DAla, and Tyr^{11} by Phe. The nature of these conformational changes is of interest and will be investigated. This "Stage 2" study has therefore been successful in enabling a much improved definition of the conformational requirements for activity.

3.5. CONCLUSION OF THE SYNTHETIC STUDY

Successful solid phase peptide syntheses have been accomplished by the continuous-flow Fmoc-polyamide method. The associated selectivity, mildness, and step-wise monitoring facilities, have been the essence of this proficient procedure.

The general application of Pfp esters with added HOBt, for coupling of Fmoc-amino acids, has proved to be as efficient as the use of Fmoc-amino acid anhydrides, whose preformation is thus avoided. The employment of Dhbt esters has also given promising results, where additional acylation monitoring is possible by visual inspection of the resin colouration. However, the use of anhydrides has been validated for the direct attachment to the resin of more sterically hindered amino acids eg. valine, due to the higher reactivity of these species.

Racemisation of cysteine has been a particularly poignant problem during esterification of this residue to the resin, when using the symmetrical anhydride as the method of activation. The epimerisation, apparently promoted by DMAP, was suppressed by reducing the amount of the base employed, and by using Pfp ester activation.

Following the first residue coupling, subsequent acylations were usually complete with a standard 25 min reaction period. However, certain residues, particularly Cys^5, Arg^9 and Val^{10}, required extended coupling periods. These sluggish reactions could be due to steric factors, which may include sequence-dependent, intra- or inter-chain, interactions.

The cleavage/deprotection of the majority of peptidyl-resins was achieved using a standard mixture of TFA-phenol-EDT. Problems were associated with peptides possessing arginine and tryptophan residues. Removal of the Mtr group from arginine required protracted treatment with the cleavage/deprotection mixture. Moreover, side product formation occurred leading to a modification of tryptophan by Mtr, or dithioketal, depending on the reaction conditions. These problems were minimised by careful monitoring of the cleavage/deprotection process.

All protecting groups, apart from the TFA stable Acm of cysteine, were removed following cleavage/deprotection. This method of cysteine

derivatisation enabled the isolation of linear peptides by preventing spontaneous disulphide bond formation.

Various cyclic peptides have been prepared for our study. The simple, mild method employing Trt protection of cysteine, cleaved by TFA, and subsequent air oxidation of the dihydro peptide, was found to be successful resulting in good yield of product. If dihydro peptides were isolated then polymerisation was found to occur and hence it is important that the controlled oxidation step should be employed as soon as possible. The value of the iodine oxidation procedure was that removal of S-protection and disulphide bond formation could be accomplished simultaneously. Cyclisation processes performed whilst the peptide remains attached to the resin should theoretically result in production of less polymeric material. Though the yield of product obtained using this procedure was slightly disappointing, further optimisation of the cleavage/deprotection step should lead to more promising results.

Homogeneous samples of each product were successfully obtained, where purification by preparative RPHPLC has proved particularly valuable.

To summarise, our synthetic study has illustrated that although solid phase peptide synthesis is facilitated by automation, sequences arise which defy facile preparation. Moreover, isolation of the product via cleavage, and subsequent purification, is by no means problem free, and requires scrupulous care. Control of first residue attachment, Mtr removal, and disulphide bond formation, was found to be particularly challenging.

3.6. FUTURE WORK - SUBSTITUTIONS AND STANDARD VARIATIONS

Our structure-conformation-activity studies have indicated key residues which are prime targets for substitution, in order to better define the exact conformational requirements for activity. To this end the replacements of Gly^8 by DAla, and Tyr^{11} by Phe, have already been carried out. The former substitution has effectively "locked" this region of the peptide in an α-helical conformation. It would indeed be interesting to perform a substitution of Gly^8 by a moiety predicted to enforce the γ-turn geometry and to determine the potency of the resulting fragment.

In the cyclic portion, where Pro^{13} is the residue responsible for the characteristic rigidity in this region, the strategy behind substitution should be to retain the rigidity while forcing this part of the peptide to adopt a different conformation. A substitution of the residue before Pro^{13}, namely Arg^{12}, may have a crucial effect. Replacement by a D-residue, or α-aminoisobutyric acid (Aib), may force this part of the peptide to adopt a type II′ β-turn or a type III β-turn (3_{10} helix). Since these are structural features which are not possible for the Arg^{12}-Pro^{13} combination, such analogues could show a pronounced effect of the backbone conformation on activity.

In addition to substitutions directed at selecting well defined, restricted conformations, substitutions can be aimed at stabilising the conformation of a residue. An example of this is Val^{10}, which shows little mobility, and occurs in the left-handed α-helical region of the (ϕ,ψ) map, which is energetically more favourable for a D-residue. Once again, substitution by a D-residue and a study of the effect could enhance our understanding of the importance of this conformational feature.

Further chemical modifications may also be made at specific points within the exocyclic residues, to test their possible binding function at the receptor molecule, or their involvement in the promotion of conformational changes. For this purpose Asp^1 and Val^{17} could be linked by an amide bond to give a bicyclic peptide.

Alterations to the stereochemistry of the active site could also be made by introducing spacer groups via $(CH_2)_2$ or $(CH_2)_3$ between the Cys-Cys linkage[100] giving larger rings, or by substitution of Cys^{14} by, for example, penicillamine,[101] thus reducing conformational flexibility near the proposed active site.

These structural alterations have established, or very recent, precedents. A tactically new variation is considered in the following Chapter, which to date has not found application in peptide modifications.

Chapter 4

THE ALLENYL *BIS*-AMINO ACIDS - A NOVEL MIMIC FOR THE DISULPHIDE BOND

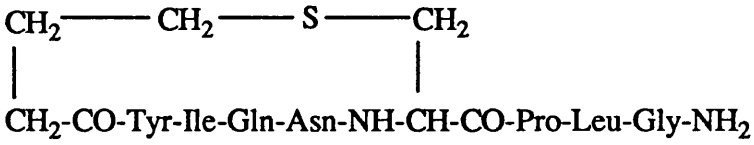
A new method of altering cyclic peptide structure and conformation is proposed for the final phase of the structure-conformation-activity study.

Our preliminary analyses have already indicated the importance of the disulphide bond for activity. A surrogate was required for this functionality, in order to allow further scrutinisation of the suggested role of the cystine bond in promoting the biologically-active conformation. Such an analogue was further desirable since the S-S bond may be susceptable to cleavage under certain conditions.

4.1. THE PROPOSED SURROGATE

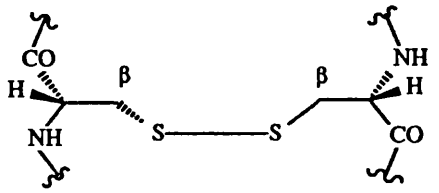
There are many reports in the literature on modifications of cystine designed to evoke conformational restrictions.[58] The introduction of alkyl spacer groups between the S-S linkage has also been proposed.[100] However, there is a distinct lack of an effective cystine substitute.

Carba-analogues of oxytocin (**53**) have been prepared as replacements for the disulphide bond,[85] and these have shown high biological activity.



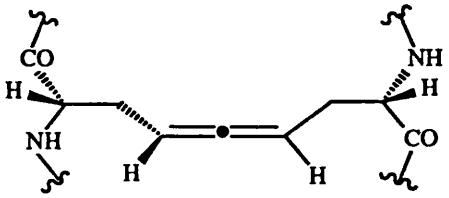
(53)

Extensive structural and conformational studies of the selenium analogue of oxytocin have also been reported,[102] in which activity was comparable to that of the native hormone. Thus, in this peptide the normal S-S bond was replaced by an Se-Se linkage. However, such substitutes do not mimic the orthogonality that a disulphide bond (**54**) elicits on the cystine β-carbons. A suitable moiety of comparable configuration was therefore sought.



(54)

It was recognised that analogous orthogonality exists in allenes. In the case of the cumulated 1,2-diene system, the central carbon atom is sp-hybridised with two sets of orthogonal p-orbitals available for overlap with the remaining p-orbitals of the two terminal sp^2 carbon atoms of the diene. For maximum overlap to occur between the p-orbitals, the resulting π-bonds must be orthogonal to each other. Thus a novel surrogate for the cystine bond could be based on the allene subunit. To this end, the allenyl *bis*-amino acid **(55)** is suitable, where there is a close correlation between the C^{β}-C^{β} distance of 4.5 Å compared to 4.0 Å for the cystine bond.



(55)

The molecular dynamics studies carried out on MCH, and the cyclic MCH(5-14) fragment, have indicated that the torsion angle around the S-S bond was approximately +90°. However, conformations with θ = -90° were not ruled out. In the proposed cystine-mimic, the allene unit can exist in two enantiomeric forms. Thus, both (R) and (S) configurations are possible giving +90° and -90° dihedral angle variability. This is of particular value, since conformational restriction is imposed, thus enabling further investigation of the effect of the torsion angle orientation on the overall conformation of the peptide.

Allenes are of widespread use as synthetic intermediates,[103] and there is a rapidly increasing number of natural products identified, containing the

allene unit.[104] Moreover, allenes have found applications in medicinal chemistry where introduction has enhanced or modified biological activity.[105] The penta-2,3-diene unit is the least space-requiring five carbon atom chain available, to serve as a rigid unit linking functional groups in probing structure-activity relationship.

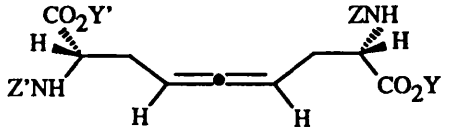
To our knowledge, the allenyl *bis*-amino acids have found no previous application in peptide modifications.

4.2. POSTULATED SYNTHETIC ROUTE

Before considering a synthetic route to the allenyl *bis*-amino acids, a suitable method for inclusion of the subunit in the peptide is required.

4.2.1. Incorporation of the Allenyl Bis-Amino Acids

Introduction of the allenyl surrogate into MCH, or a related analogue, may be accomplished by the Fmoc-polyamide solid phase methodology. However, for this purpose, each of the amino acids must be suitably functionalised yet differentiated. To this end the derivatised product (**56**) may be suitable.

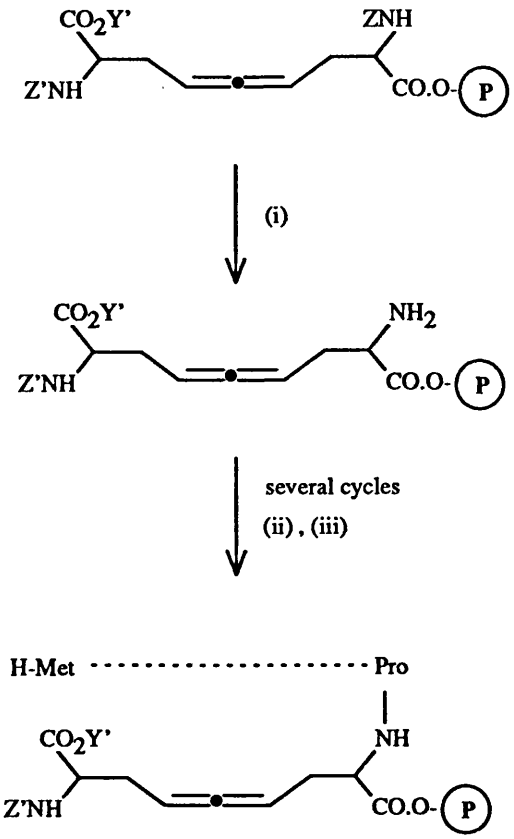


Z = Fmoc ; Z' = Dts ; Y = Pfp ; Y'= ONb

(56)

It is proposed that (**56**) would be most readily incorporated into the cyclic MCH(5-14) fragment. The solid phase procedure is outlined in **Scheme 13**. The allenyl subunit may be directly attached to the benzyl alcohol functionalised resin via the Pfp ester functionality (Y). On cleavage of the Fmoc group (Z), the next amino acid of the sequence, proline could then be coupled to the free amine in the usual fashion. The synthesis would then be continued until the complete 6-13 sequence of MCH had been assembled. The protecting groups, Dts and ONb, employed

for the amine, and carboxylic acid functionalities of the other amino acid of the allenyl unit, should remain stable to the conditions of the synthesis.

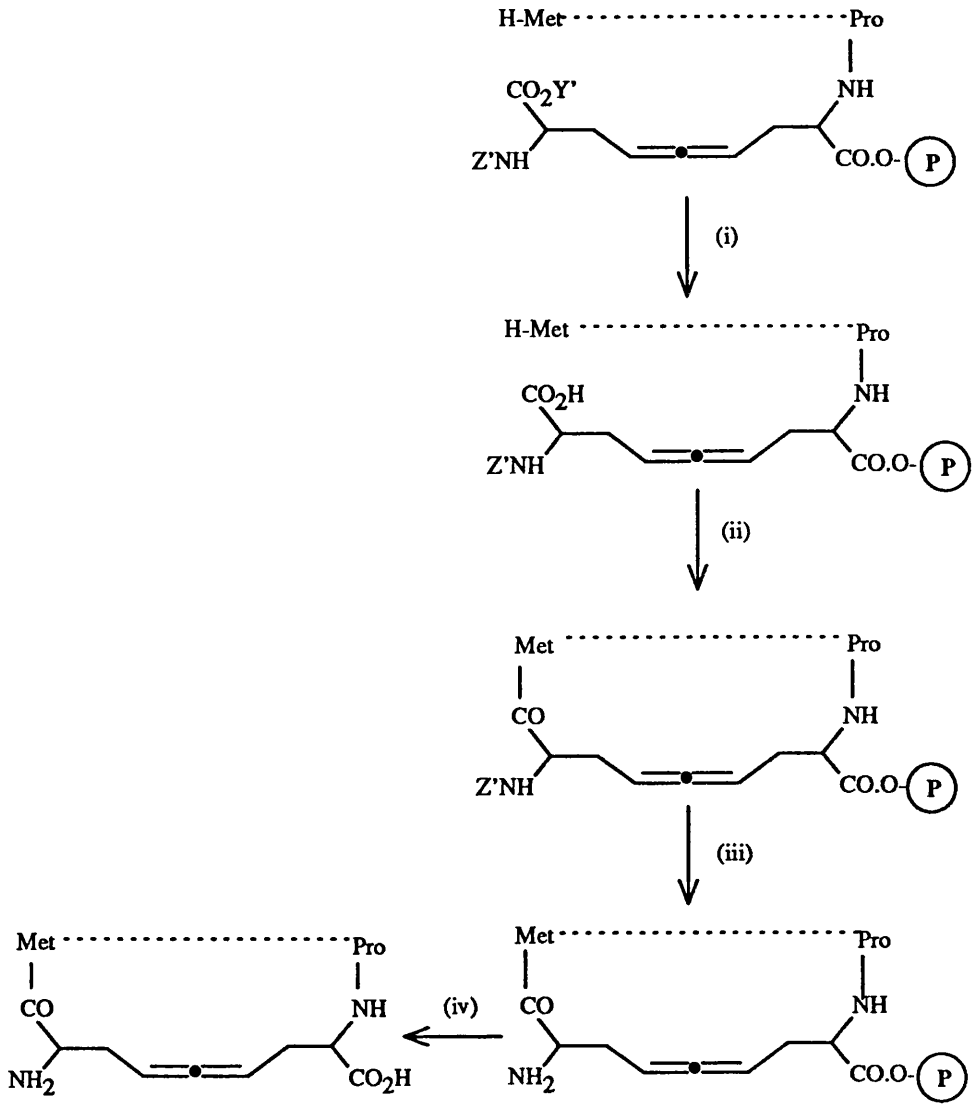


(i) 20% piperidine-DMF (ii) Fmoc-amino acid : Pfp ester , HOBt

(iii) 20% piperidine-DMF

Scheme 13

The required cyclisation **(Scheme 14)** could be performed on the resin by the active azide procedure. However, cleavage of the ONb protection is first required and may be elicited by photolysis at hv:350 nm. Finally, removal of the Dts group is effected by thiols, and treatment with TFA and appropriate scavengers will cleave the peptide from the resin and remove remaining side-chain protection. This would yield the desired cyclic subunit where the allenyl moiety is a substitute for the Cys-Cys functionality.

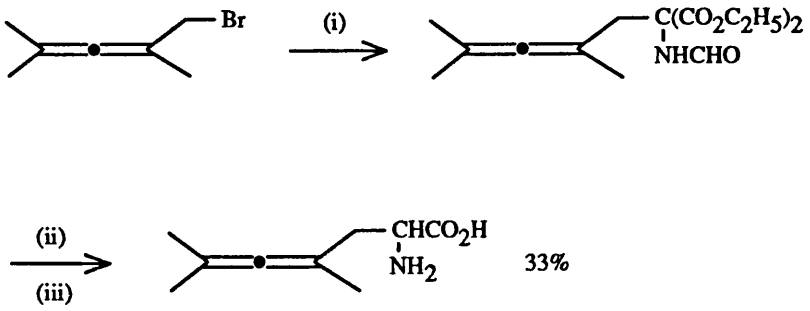


(i) hv : 350 nm (ii) DPPA- HOBt (iii) RSH (iv) TFA , scavengers

Scheme 14

4.2.2. Preparation of the Allenyl *Bis*-Amino Acids

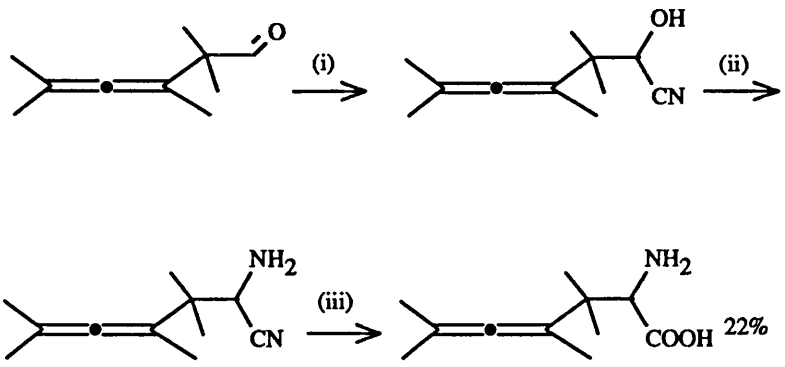
General methods are available for the preparation of racemic β-allenic α-amino acids. One procedure involves treatment of bromoalkadienes with diethyl formylaminomalonate (DFAM), using sodium hydride as the condensing agent,[106] as shown in **Scheme 15**. The (S) enantiomer of the resultant compound, 2(S)-aminohexa-4,5-dienoic acid, is a natural product.[107]



(i) NaH , DFAM (ii) NaOH (iii) HCl

Scheme 15

Another route is based on the conversion of allenic aldehydes into allenic amino acids by a modified Strecker synthesis.[108] However, moderate yields of product are obtained only from allenic aldehydes with blocking of the 2-position **(Scheme 16)**, since unsubstituted 3,4-dienals tend to rearrange under the alkaline reaction conditions to 2,4-dienals.



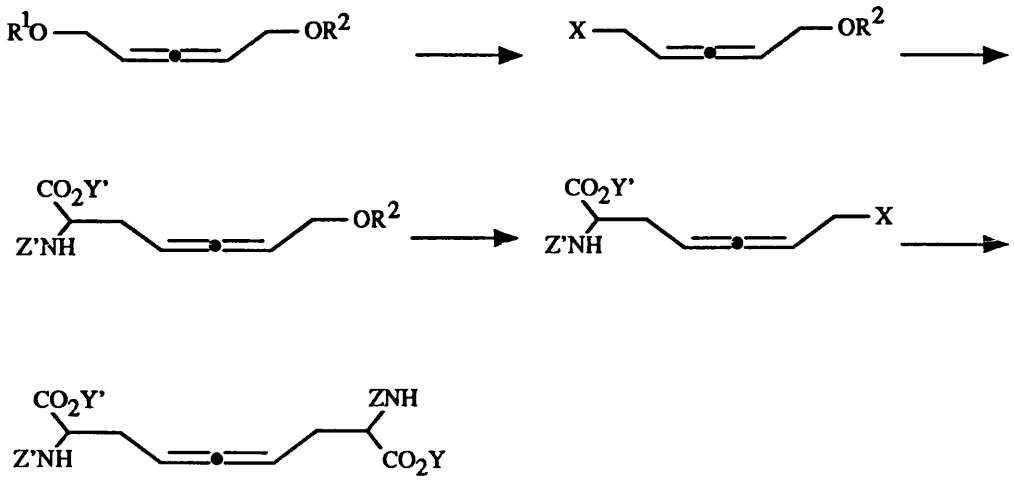
(i) HCN , KCN , 0°C (ii) NH_4Cl - NH_4OH , 20°C , 7 days (iii) HCl

Scheme 16

The α-allenyl α-amino acids are of interest, due to their envisaged biological properties, and methods have been established for their preparation.[109]

As yet however there is no reported synthetic procedure for the allenyl *bis*-amino acids. The (S,S)-diastereomers of the (R) and (S) allenes are required in order to maintain the natural amino acid chirality. Possible

precursors to the derivatised moiety (**56**) are outlined in **Scheme 17.**



(56)

Scheme 17

Thus, starting from the allenic diol(R^1,R^2=H), mono-protection enables subsequent distinction of each amino acid introduced. The remaining free hydroxyl may then be activated (possibly by conversion to a halogen), to enable alkylation of a glycine enolate (α-anion synthon). This may be achieved stereoselectively by applying the methodology of Schöllkopf ,[110] whose general procedure utilises a bis-lactim ether derived from valine and glycine. The appropriate protection of the amino and carboxyl groups, as Dts and ONb respectively, may then be introduced on liberating the amino acid moiety of the desired chirality.

The other amino acid of the allenyl unit may then be incorporated in a similar fashion following deprotection of the masked hydroxyl. It is anticipated that the allene, Dts, and ONb functionalities will remain intact under the hydrolysis conditions required to liberate the amino acid, which can then be derivatised as the Fmoc protected Pfp ester.

Other stereoselective methods for synthesis of α-amino acids may also be envisaged, based on electrophilic amination of the corresponding enolate.[111] Moreover, asymmetric synthesis on carbohydrate templates,[112] and asymmetric addition using a CN-modified Hemin-copolymer[113] are recently reported methods of interest.

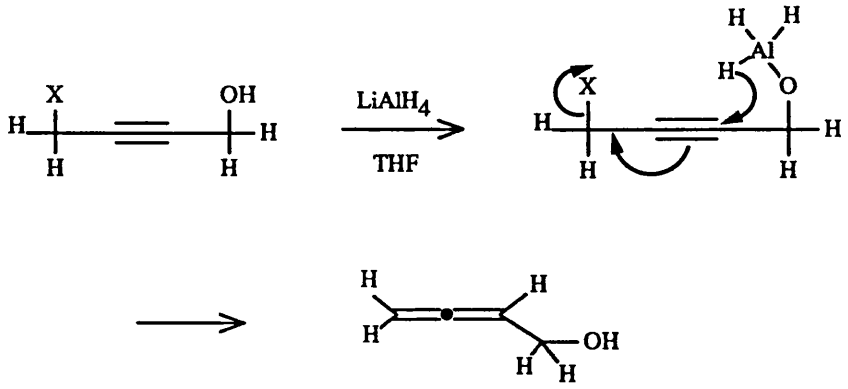
Thus the allenic diol was selected as the initial target compound.

4.3. SYNTHESIS OF 1,5-DIHYDROXYPENTA-2,3-DIENE

A survey of the literature revealed that the allenic diol (57) was an unknown compound. A facile synthesis of this important starting material was therefore required.

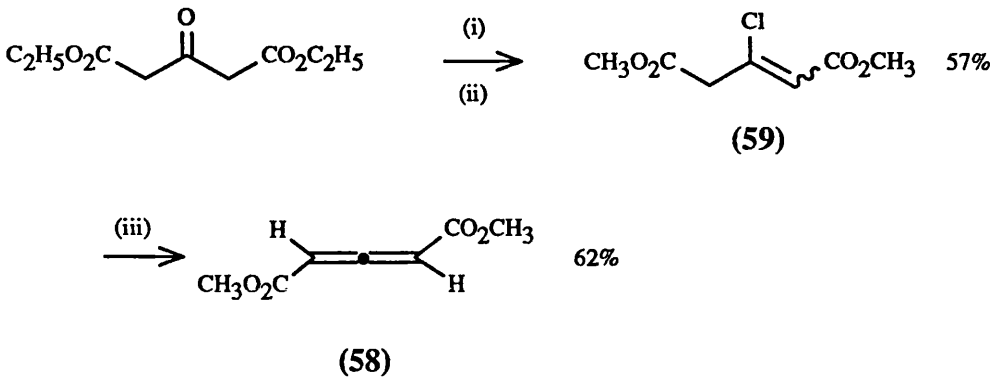
(57)

A generally reliable method for the preparation of allenic alcohols is the addition of lithium aluminium hydride to propargylic alcohols[114] as shown in **Scheme 18.**



Scheme 18

However, a simple route involving the reduction of the known, corresponding, diester allene was envisaged. Synthesis of dimethyl penta-2,3-dienedioate (58) was accomplished according to the procedure of Bryson et al,[115] **(Scheme 19).** An alternative method involves the intermediate enol phosphate.[116]



(i) PCl_5 , 25°C (ii) H_2SO_4 , MeOH , reflux , 18 h (iii) TEA , THF , 0 - 5°C , 18 h

Scheme 19

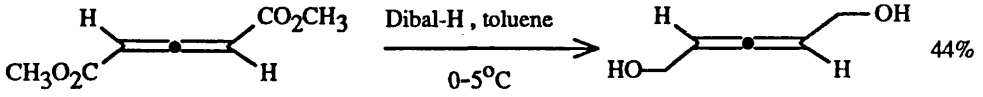
The product **(58)** was purified by distillation, with higher yields being obtained by performing this process in small batches. The compound required storage at low temperature to minimise polymerisation processes, detected by the increased viscosity and colouration. The strong i.r. absorption detected at 1960 cm^{-1} was characteristic of the allenic antisymmetric stretch. In addition, the extreme low field position of the allene central carbon was identified by ^{13}C n.m.r. spectroscopy at δ 219.5 ppm. Having obtained **(58)** in reasonable yield, a suitable method of reduction was sought.

Initial attempts by treatment with $LiAlH_4$ were unsuccessful. The reaction was performed in ether, at various sub-zero temperature levels, but on each occasion no allene-containing products could be detected by i.r. spectroscopy, where the antisymmetric stretch at *ca.* 1950 cm^{-1} was absent. However, a strong OH peak was present and there was a lack of any carbonyl stretching frequency. Further examination by ^1H n.m.r. spectroscopy revealed the possibility that a mixture of 1,5-pentanediol and vinyl adducts had been formed.

Though it is generally accepted that sodium borohydride will not reduce carboxylic esters, cases are reported in the literature in which reduction of esters to primary alcohols has been observed.[117] Many of the compounds which undergo such a reduction contain neighbouring functional groups which may participate in some way. A reduction with

$NaBH_4$ was attempted, in aqueous ethanol at 0°C. This reagent indeed effected reduction of the ester groups, but spectroscopic evidence indicated that reduction of the allene unit had also occurred.

The required reduction was finally achieved by employing diisobutylaluminium hydride (Dibal-H) as the reducing agent, as shown in **Scheme 20.**



(58) (57)

Scheme 20

It was found that decomposition of the aluminium salts was best achieved by treatment of the intermediate aluminium complex with potassium sodium tartrate solution, followed by a continuous extraction into ether to isolate the product. Use of $MeOH/H_2O$ as the initial treatment resulted in lower yield of product. Purification was best effected by column chromatography to furnish **(57)** in 44% yield. Use of distillation, even of small batches, resulted in extensive polymerisation and decomposition of the diol; this also occurred on standing at RT ,but was suppressed by storage at low temperature. Only a small proportion (*ca.* 5%) of the totally reduced product 1,5-pentanediol was isolated.

The identity of **(57)** was confirmed from spectral data, which showed strong absorptions in the i.r. spectrum at 3600-3100 cm^{-1} (OH) and 1970 cm^{-1} (allene), with no carbonyl absorption detected. In the ^1H n.m.r. spectrum no coupling was detected from the C-2 to C-4 proton or from C-1 to C-5 protons due to the symmetry of the molecule (see **Figure 31**). Coupling of C-1 protons to those of the adjacent C-2 and the long-range proton-proton coupling, ^5J (H-H) to C-4 protons was identical, giving rise to a triplet at δ 4.16 ppm. Similarly, coupling of the C-2 proton to the protons of C-1 and C-5 elicits a triplet at δ 5.51 ppm. The allenic central carbon was evident in the ^{13}C n.m.r. spectrum at δ 202.5 ppm. Furthermore, mass spectrometric analysis by chemical ionisation (C.I.) revealed a weak $(M+1)^+$ peak at m/z 101 with an intense signal at m/z 83

$(M+1-H_2O)^+$. Due to decomposition of the product a satisfactory elemental analysis result remained unobtainable.

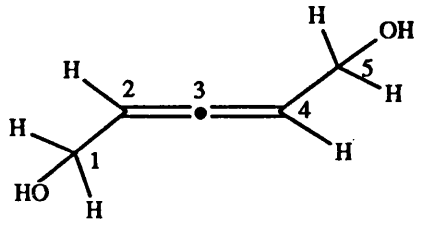


Figure 31

Treatment of allenes with Dibal-H has been reported, where reduction of the more substituted double bond of the allenic linkage has been selectively achieved.[118] However, in our system, Dibal-H has proved to be an effective reagent for the conversion of allenic ester to allenic alcohol, without affecting the integrity of the allene moiety to any great extent.

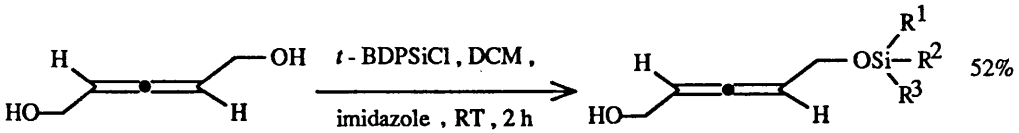
4.4. SYNTHESIS OF 5-(*T*-BUTYLDIPHENYLSILYLOXY)PENTA-2,3-DIENE-1-OL

Having obtained the allene diol (**57**), a method for selective protection was required.

The utility of *t*-butyldiphenylsilyl chloride (*t*-BDPSiCl)[119] as a reagent for the protection of hydroxyl groups was explored. The ease of formation, adaptability to various analytical techniques, and compatibility with a variety of conditions encouraged the utility of the *t*-BDPSi ether. In addition, they have greater stability to acids than the related silyl and butyl ethers. This is desirable when protection is required to be maintained such as on acid hydrolysis reactions to yield the amino acid moiety at the other C-terminus of the allene unit. However, smooth cleavage is effected by treatment with fluoride ion.

The formation of the mono *t*-BDPSi ether was attempted by treatment of (**57**) with *t*-BDPSiCl and NaH in THF. However, after several attempts, no allenic product could be detected and hence the procedure was abandoned.

However, the desired compound **(60)** was obtained by use of imidazole in place of NaH according to **Scheme 21.**



(57) **(60)**

$R^1 = (CH_3)_3C$; R^2 , $R^3 = C_6H_5$

Scheme 21

A portion of the disilylated product (*ca.* 10%) was also obtained.

All spectral data were consistent with the desired product. An OH band was present in the i.r. spectrum at 3600-3100 cm^{-1}, with additional bands at 1100 and 820 cm^{-1} (Si-O-C). The presence of the *t*-BDPSi ether was indicated by *t*-Bu and aromatic signals in the ^1H and ^{13}C n.m.r. spectra.

The mono-protection renders loss of symmetry, and this was shown by ^1H n.m.r. which revealed a doublet of doublets at δ 4.08 and 4.25 ppm for the protons of the hydroxy- and silyloxy-methines respectively, due to ^3J (H-H) and ^5J (H-H) coupling in each case. Each allenic proton was now differentiated giving rise to a complex signal (theoretically of eighteen lines). Mass spectrometry (C.I.) revealed a major signal at m/z 321 due to loss of H_2O from the molecule, in addition to the $(M+1)^+$ peak at m/z 339.

The compound **(60)** was more stable than the non-functionalised allenic diol and polymerisation no longer took place.

4.5. ATTEMPTS TO ACTIVATE THE ALLENE MOIETY FOR ALKYLATION

Various methods of activation, for subsequent alkylation reactions were applicable.

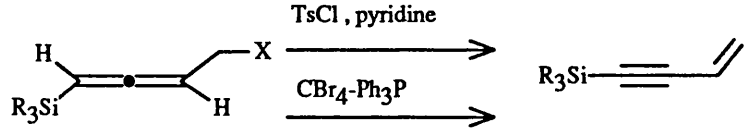
Initially efforts were concentrated on the allenic diol **(57)** as a model

compound. The successful reactions may then be performed on the mono-protected derivative **(60)**.

However, many attempts to obtain the difunctionalised diol were unsuccessful. The ditosylated adduct was targeted and the allenic diol **(57)** was thus treated with tosyl chloride in pyridine. The tosyl groups might then be displaced by iodo functionalities to give halogen activation, if indeed alkylation could not be performed directly on the tosyl derivative. However, only a mixture of tosylated and vinylic material was obtained, devoid of any allenic product, as indicated by i.r. and n.m.r. spectroscopy.

The direct bromination of the allenic diol **(57)** was also attempted. The preparation of allenic bromides by treatment with triphenyl phosphite dibromide[120] has been reported, but in our case monitoring of the reaction by i.r. and ^1H n.m.r. spectroscopy revealed that destruction of the allenic moiety had again occurred. Similarly, treatment of **(57)** with CBr_4-PPh_3[121] yielded uncharacterised vinylic product.

Thus, the derivatisation of **(57)** was not attainable by several standard procedures. It appears that activation of the alcohol moieties has increased the sensitivity toward decomposition as evident by its rapid destruction. Such activation may have caused the system to become extremely prone to elimination. Indeed, similar work has been carried out by Trost et al[122] on the activation of α-silylated β-hydroxy allenes. Attempts at tosylation and bromination resulted in the exclusive formation of the elimination product **(Scheme 22)**.

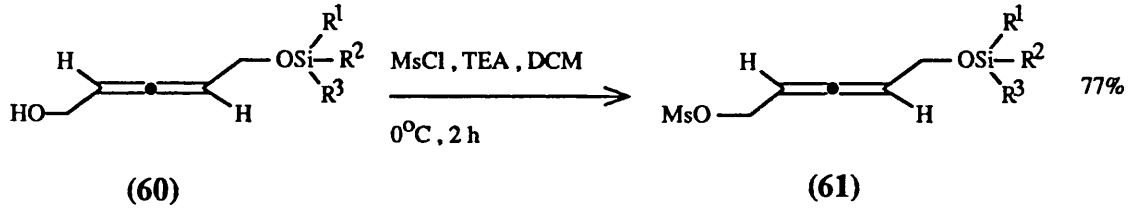


Scheme 22

An analogous process may also have occurred in our case, though no i.r. absorption bands, characteristic of acetylene, were detected from reaction products.

Finally, attempts to activate the allenic diol **(57)**, by formation of the

dimesylated derivative, appeared to have been successful. Therefore, we treated the mono-protected diol (**60**) similarly, according to **Scheme 23**, to obtain the mono-silylated, mono-mesylated product (**61**). The i.r. spectrum revealed absence of the OH band and the appearance of signals at 1340 and 1160 cm^{-1}, due to the sulphonate ester. Presence of this group was further revealed by the methyl singlet at δ 1.05 ppm in the ^{1}H n.m.r, and at δ 38.3 ppm in the ^{13}C n.m.r. spectra. The allene was identified by the usual characteristic signals in the i.r. and ^{13}C n.m.r. analyses. Moreover, the mass spectrum (C.I.) showed peaks due to fragments where losses of sulphonate ester moieties had occurred, at m/z 338 $(M+1-SO_2CH_3)^+$ and 321 $(M-OSO_2CH_3)^+$. Attempts to purify the product (**61**) by column chromatography resulted in its decomposition. The compound again required storage at low temperature.



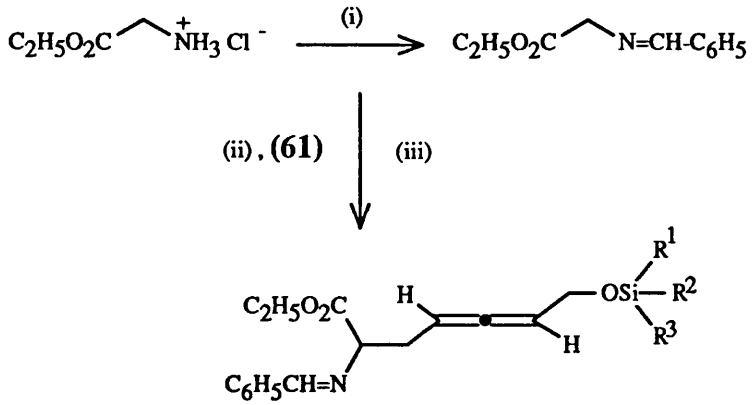
(**60**) (**61**)

Scheme 23

Thus, the mono-protected, activated allene was obtained as required. There appears to be no obvious explanation for the success of the mesylation activation over the other procedures attempted. A model alkylation study was then carried out, designed to test the suitability of the mesylate (**61**) for such processes.

It was recognised that benzylidene derivatives of glycine can be readily alkylated to provide a simple route to α-amino acids.[123] Thus we attempted this reaction according to **Scheme 24**, using (**61**) as the alkylating species.

Our results indicated that after several hours reaction period, the yellow colour of the anion remained and starting material **(61)** was still evident by t.l.c. and ^1H n.m.r. spectroscopy. Traces of a new product were detected by these methods which may be attributed to the desired alkylated species. However, due to the small scale of the reaction this was not isolated, and the result remains inconclusive.



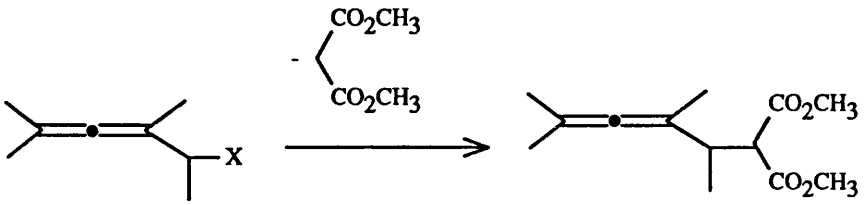
(i) C_6H_5CHO, DCM, TEA, $MgSO_4$, RT, 15 h (ii) KO-*t*-Bu, THF, -78°C

(iii) **(61)**, -78°C→ RT, several hours

Scheme 24

4.6. SUMMARY OF THE CYSTINE SURROGATE SYNTHETIC STUDY

Our results have indicated that the mesylate may not be an appropriate leaving group for the required alkylation reaction, and hence alternative methods must be pursued. Previous attempts to dibrominate the dihydroxy allene failed, but bromination or iodination may prove to be effective on the mono-protected allenic hydroxy compound **(60)**, or indeed on the mesylate **(61)**. Alternatively, palladium catalysed alkylation methodology may be employed on the benzylidene glycine ethyl ester as has been applied in other alkylation reactions[124] **(Scheme 25)**. It appears however that problems due to instability of intermediates must be overcome.



X = OAc , O-PO-$(OC_2H_5)_2$

Scheme 25

Once a suitable method of activation for alkylation has been achieved for this model system, the synthesis of the suitably functionalised allenyl *bis*-amino acids **(56)** may be in prospect. Once incorporated into MCH, or a related analogue, as a cystine mimic, resolution of the diastereomeric peptides (where (R) and (S) allenic configurations are possible) may be achieved by application of RPHPLC. The subsequent structure-activity study, and n.m.r. analysis of conformation, will enable further investigation of the effect of the disulphide functionality on the biologically active conformation of MCH.

CONCLUSION

Final Conclusion of the Synthetic and Structure-Conformation-Activity Studies of MCH

Our synthetic studies of MCH and related analogues have enabled us to gain knowledge of the preparation of synthetically challenging sequences. The Fmoc-polyamide solid phase approach was used effectively for this purpose. It is hoped that our findings may find useful applications in future preparations to overcome some of the difficulties.

Our overall aim was to elucidate the essential structural and conformational features of MCH that are required for activity. The results from the "Stage 1" and "Stage 2" studies obtained thus far, have enabled some understanding of these aspects. To further probe the importance of the disulphide bond, we have also commenced the synthesis of an unprecedented cystine substrate.

Our findings have suggested that peptides of the size of MCH may possess more structural and conformational integrity than could originally be anticipated. The study of this interesting molecule has indeed been justified. Further synthetic studies may be envisaged, designed to induce further conformational restrictions. The subsequent analysis of conformation, and assessment of biological activity, should lead to an increased understanding of the receptor site requirements of MCH.

EXPERIMENTAL

Experimental

MATERIALS AND METHODS

Peptide Syntheses

Solvents and Techniques

All solid phase syntheses were carried out on a semi-automatic Pepsynthesiser apparatus from Cambridge Research Biochemicals (CRB), fitted with two glass reaction columns (83 mm x 10 mm diameter). The various steps were monitored continuously by u.v. spectrophotometry at 330 nm, at a flow rate of 3 cm^3 per minute. The 4-hydroxymethylphenoxyacetyl-norleucyl-derivatised-kieselguhr supported resin (Pepsyn KA), was a CRB product, as were all the Fmoc-amino acids and active esters.

Peptides were isolated from aqueous solutions by lyophilisation, after removal of excess organic solvents using a Büchi rotary evaporator employing water aspiration to avoid heating. All amino acid derivatives, and peptide products were stored at -20°C. All resins and peptidyl-resins were stored at 4°C.

N,N-Dimethylformamide (DMF) was freshly, fractionally distilled under reduced pressure and both dichloromethane (DCM) and diethyl ether were purified immediately before use by percolation through active basic alumina. 1-Hydroxybenzotriazole (HOBt) was recrystallised from hot water.

Trifluoroacetic acid (TFA), for reverse phase high performance liquid chromatography (RPHPLC), was purified by distillation from solid potassium permanganate. Water was double-distilled-de-ionised, and acetonitrile was far-u.v. transparent HPLC grade. All solutions were filtered through a Millipore membrane before use.

Unless otherwise stated, yields are quoted based on the theoretical amount of peptide that was expected, assuming 100% resin coverage and quantitative acylations during the solid phase assembly. For some cyclisation reactions, yields relate to the precursor linear peptide.

Volumes of reagents used that were less than 0.5 cm^3, are quoted as the appropriate value in units of µl (microlitres).

Chromatography

Gel permeation chromatography was performed on a column (19 cm x 3 cm diameter) of Bio-Gel P-2 (Bio-Rad, exclusion limit 2000 Daltons), packed in an aqueous solution of 1 *M* acetic acid and eluted with the same solution. Alternatively, a column (19 cm x 3 cm diameter) of Sephadex LH20 (Pharmacia) packed in and eluted with an aqueous solution of 1% acetic acid was utilised. In both cases, the eluting solutions were degassed before use and the effluent monitored at 254 nm, at a flow rate of 0.5 cm^3 per min. Any deviations from these procedures are noted.

Unless otherwise stated, RPHPLC was performed in the analytical mode on a 5μ C_{18} Spherisorb column (250 mm x 4.6 mm diameter), at a flow rate of 1 cm^3 per min. It was used extensively to monitor reactions and as a determination of product purity. Preparative RPHPLC was carried out on a 5μ C_{18} Hypersil column (250 mm x 10 mm), at a flow rate of 4 cm^3 per min. In both the analytical and preparative modes, gradients were generally formed between the degassed solvents, A (0.1% TFA in H_2O), and B (0.1% TFA in CH_3CN-H_2O 9 :1 v/v), using a profile of 5 min at 5% B, followed by a linear gradient of 2% per min to 100% B. Peak detection was carried out at 214 nm. The % B at which the product peak appeared is quoted, together with the retention time (t_R).

Thin layer chromatography (t.l.c.), on Merk DC-alufolien plates, coated with Kiesel gel 60 F_{254}, was also used to establish homogeneity of products. Three general solvent systems were adopted:

(S1) butan-1-ol-acetic acid-pyridine-water (4:1:1:2 v/v)
(S2) butan-1-ol-acetic acid-pyridine-water (5:1:3:5:4 v/v)
(S3) propan-1-ol-pyridine-water-acetic acid-ethyl acetate (5:4:6:1:4 v/v)

Visualisation was achieved by spraying with a solution of 1% ninhydrin in butan-1-ol (w/v), followed by gentle heating. The retention index (R_f) is quoted for each peptide.

Amino Acid Analyses

Amino acid analyses were performed on samples hydrolysed at 120°C for 18 h, using a modification of the Waters "Picotag" system,[125] on a 250 mm x 4.6 mm Spherisorb ODS2 column, using two Waters 510 delivery systems. The solvents used were:

(A) 0.14 *M* sodium acetate (0.85 cm^3) in triethylamine (TEA) (1000 cm^3), pH 5.6

(B) CH_3CH-H_2O (6:4 v/v)

The profile consisted of 0% B for 2 min, followed by a convex gradient to 42% B over 15.5 min, and then to 100% B over 4 min.

Amino acid ratios are quoted based on valine as the reference.

Spectroscopy

Fast atom bombardment (F.A.B.) mass spectrometry was routinely applied to confirm the composition of each peptide. The molecular protonated species, $(M+H)^+$, is quoted. Generally, matrices constituted glycerol, diglycerol and thioglycerol, often in various mixtures, and Xenon was used as the bombarding agent.

Nuclear magnetic resonance (n.m.r.) spectra were measured using the manufacturer's standard spin sequences. 2D spectra were measured using a 1024 x 256 matrix with zero-filling on the f_1 axis, and at 30°C to maintain temperature stability. Long range correlation spectroscopy (LRCOSY) refers to a COSY sequence with a fixed delay in the evolution period. Nuclear Overhauser effect (NOE) enhancements were detected by 2D NOE (NOESY) measurements using 100, 300, and 500 millisecond mixing time on each occasion, then measured using an NOE difference sequence with homo-gated decoupling applied at indicated signals. Solvent suppression was achieved, where necessary, by selective saturation.

Instrumentation

^{1}H N.m.r.	Jeol GX FT 400 (400 MHz)
RPHPLC	Apparatus A: Gilson 303 pumps, LDC/Milton Roy variable wavelength u.v. detector, Apple microprocessor controller.
	Apparatus B: LKB 2150 pumps, 2140 diode array detector, 2152 controller, 2157 autosampler
F.A.B.	VG 7070E with 2000 data system
	High resolution ZAB-E

Other Preparations

Solvents and Techniques

Petroleum ether (petrol) refers to petroleum spirit, b.p. range 60-80°C, and ether refers to diethyl ether. Reaction solvents were dried and distilled before use, as were solvents used for chromatography. Tetrahydrofuran (THF) was pre-dried over sodium wire, and then refluxed over sodium benzophenone ketyl until dry, and re-distilled prior to use. DCM and TEA were dried by distillation from calcium hydride, and methanol (MeOH) and ethanol (EtOH) were distilled from magnesium turnings.

For reactions performed under dry nitrogen, the glassware and apparatus were dried overnight in an oven at 125°C, then allowed to cool in a dessicator. Flasks were sealed with rubber septums and flame dried before use. All reactants were transferred with syringes and needles.

All yields quoted are of purified products, unless otherwise stated.

Chromatography

Reactions were monitored by t.l.c., on Merk DC-alufolien plates coated with Kiesel gel 60 F_{254}. Visualisation of reaction components was achieved by illumination under short wavelength (254 nm) ultraviolet light and/or spraying with any one of:

(a) 7% (w/v) methanolic solution of phosphomolybdic acid (PMA)

(b) 0.5% (w/v) aqueous potassium permanganate solution

(c) 1% ninhydrin in butan-1-ol (w/v)

Column chromatography was performed using short path pressurised columns packed with silica gel (Merk 7747), prepared in petrol, then eluted with petrol-ethyl acetate, increasing the polarity. Mixtures to be separated were pre-absorbed onto the column support, or introduced as a thin layer dissolved in the eluant solution.

Spectroscopy

N.m.r. spectra were recorded at 270MHz (^1H), or 67.8 MHz (^{13}C), and were run in deuteriochloroform ($CDCl_3$) with tetramethylsilane (TMS) as internal standard, unless otherwise stated. Chemical shifts (δ) are expressed as downfield shifts from TMS with multiplicities denoted by s (singlet), d (doublet), t (triplet), q (quartet), p (pentet), dd (doublet of doublets), dt (doublet of triplets), bs (broad singlet), m (multiplet), bm (broad multiplet) and c (complex). Infrared (i.r.) spectra were recorded as liquid film or nujol mulls, with the absorption frequencies (υ) expressed in cm^{-1}. Mass spectra (m.s.) were recorded using electron impact (E.I.) and/or chemical ionisation (C.I., reagent gas isobutane) techniques, quoting the molecular ion $(M)^+$ and/or molecular protonated ion $(M+1)^+$ respectively. In addition, the base peak and sizeable fragmentations are denoted with assignments, where possible. All melting points (m.p.s) are uncorrected. Elemental microanalyses were obtained from the Physical and Chemical Measurement Unit (University of Bath).

Instrumentation

^{1}H N.m.r.	Jeol GX FT 270 (270 MHz)
	Varian EM-360 (60MHz)
	Hitachi Perkin-Elmer R24 (60MHz)
^{13}C N.m.r.	Jeol GX FT 270 (67.8 MHz)
I.r.	Perkin-Elmer 197 and 1310 grating
M.s.	VG 7070E with 2000 data system
M.p.	Electrothermal MKII

SOLID PHASE PEPTIDE SYNTHESES - THE GENERAL PROCEDURE

The following general procedure was followed for the synthesis of each peptide unless otherwise stated.

All solid phase peptide assemblies were carried out using the continuous-flow 9-fluorenylmethyloxycarbonyl (Fmoc)- polyamide method.[24]

The 4-hydroxymethylphenoxyacetyl-norleucyl-derivatised polydimethylacrylamide-kieselguhr supported resin (Pepsyn KA) was used for each synthesis. The resin was allowed to swell in DMF and washed clear of fine material by several cycles of gentle swirling and decantation before being transferred in DMF to the reaction column(s) of the synthesiser.

The first amino acid of the sequence was esterified to the resin bound linkage agent using a 4-fold excess of either (a) the freshly prepared symmetrical anhydride or (b) the pentafluorophenyl (Pfp) ester, of the Fmoc-amino acid to be coupled in the presence of 4-*N*,*N*-dimethylaminopyridine (DMAP). Unless otherwise stated, all Fmoc-amino acid anhydrides were prepared from the corresponding Fmoc-amino acids (8-fold excess) by the addition of *N*,*N*'-dicyclohexylcarbodiimide (DCC) (95% molar equivalents) to a solution of the Fmoc-amino acid in DCM. After stirring at RT (*ca.* 10 min) the precipitated *N*,*N*'-dicyclohexylurea (DCU) was removed by filtration and the filtrate evaporated to dryness under reduced pressure to afford the anhydride which was then used immediately. In general, the quantity of DMAP used was 0.1 molar equivalents of the number of moles of amino acid derivative employed. For incorporation, a solution of the Fmoc-amino acid derivative in DMF (*ca.* 3 cm^3) was added to the top of the resin bed and rinsed on with DMF. A solution of DMAP in DMF (*ca.* 0.5 cm^3) was then added and the recirculation mode entered as soon as the reactants were detected to have passed through the resin. After a certain recirculation period usually (a) 50 min for anhydrides and (b) 2 h for Pfp esters, the system was washed with DMF and a further batch of reactants employed in the same manner to ensure a high level of esterification of the resin active sites. This was monitored by the application of a quantitative ninhydrin colour test.[45] In some cases a quantitative Fmoc test was employed (both tests are described subsequently). If the extent of resin coverage was low then a further batch of reactants was added.

Having acylated the resin active sites satisfactorily, the Fmoc group was cleaved with 20% piperidine-DMF (v/v). In general, exposure to piperidine-DMF was for a 15 min period. The synthesis was then continued with repetitive cycles of

acylation and Fmoc cleavage. Fmoc-amino acids were introduced in a stepwise fashion as part of a cycle using a 4-fold excess of the activated derivative. In general Pfp esters were used with the simultaneous addition of a molar equivalent of HOBt. Freshly prepared symmetrical anhydrides were also employed and in some cases 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine (Dhbt) esters were utilised. On addition of the Dhbt ester of the Fmoc-amino acid, a yellow colouration appeared on the resin which was observed to fade as the acylation proceeded. All reactants were added as solutions in the minimum volume of DMF (*ca.* 3 cm^3) and were recirculated through the resin in the usual fashion for an initial 25 min period. A qualitative ninhydrin colour test,[43] (Kaiser test - described later) was then employed to check for a complete acylation reaction. This test is specific for primary amino groups, hence an extended acylation period (120 min) was automatically allowed for a coupling reaction when the amino terminal residue was proline. If an acylation reaction was judged to be incomplete, then an extended recirculation period was allowed until a satisfactory Kaiser test resulted (alternatively a fresh batch of reactant was employed when using anhydrides as the acylating species). On confirmation of complete reaction, the intermediate Fmoc-peptidyl-resin was treated with 20% piperidine-DMF to effect removal of the Fmoc group. On culmination of the synthesis, and removal of the Fmoc group, the peptidyl-resin was sequentially washed with DMF, *t*-amyl alcohol, glacial acetic acid, *t*-amyl alcohol, DCM and ether, using *ca.* 50 cm^3 of each per gram of peptidyl-resin, which was then dried in a stream of nitrogen and stored at 4°C.

Details of the Pepsynthesiser II Synthetic Cycles

Under FLOW conditions the solvent/reagent selected was pumped through the reaction column(s) and u.v. flow cell and out to waste. All washing of resin (with DMF, REAGENT A), deprotection (treatment with 20% piperidine-DMF, REAGENT B) and loading of reactants or DMF from the sample syringe (S) were carried out in this way. In the RECIRCULATE mode, the solution was continuously recycled through the reaction columns and flow cell; this was the situation during coupling of an Fmoc-amino acid. During CALL (indefinite period) the appropriate colour test was carried out.

A programme for the incorporation of the first Fmoc-amino acid consisted of the following operations:

Step Number	Operation	Time/min
1	FLOW A	15
2	FLOW S (reactants)	1
3	RECIRCULATE (coupling)	(a) 50 (for anhydride) (b) 120 (for Pfp ester)
4	FLOW S (DMF)	1
5	FLOW A	5
6	RECIRCULATE	0.5
7	FLOW A	5
8	FLOW S (reactants)	1
9	RECIRCULATE (coupling)	(a) 50 (for anhydride) (b) 120 (for Pfp)
10	CALL	
11	FLOW S (DMF)	1
12	FLOW A	5
13	RECIRCULATE	0.5
14	FLOW A	10

Steps 8-14 were repeated if resin coverage was shown to be low.

A programme for the incorporation of each remaining Fmoc-amino acid consisted of the following:

Step Number	Operation	Time/min
1	FLOW A	5
2	FLOW B	15
3	FLOW A	15
4	FLOW S (reactants)	1
5	RECIRCULATE	25
6	CALL	
7	RECIRCULATE	25
8	FLOW S (DMF)	1
9	FLOW A	5
10	RECIRCULATE	0.5
11	FLOW A	5

Steps 5 and 6 were repeated if acylation is incomplete.

Procedures Adopted For The Various Colour Tests

Quantitative Determinations

(i) **Quantitative Ninhydrin Colour Test**

The following solutions were prepared:
aqueous KCN (0.01 *M*, 2 cm^3) in pyridine (98 cm^3) added to a solution of phenol (40.0 g) in EtOH (10 cm^3); ninhydrin (2.5 g) in EtOH (50 cm^3).

The acylated resin (*ca.* 2 mg) was removed from the reaction column and placed in a sintered glass filter tube. A solution of 20% piperidine-DMF was passed through the column (3 x 5 cm^3), followed by washes with DMF (3 x 5 cm^3), *t*-amyl alcohol (3 x 5 cm^3), glacial acetic acid (3 x 5 cm^3), *t*-amyl alcohol (3 x 5 cm^3), DCM (3 x 5 cm^3) and ether (4 x 5 cm^3). The acylated resin was dried in a stream of nitrogen and the weight determined accurately. After transferance to a small ignition tube the solution of KCN and phenol (100 µl) was added followed by the solution of ninhydrin (25 µl). These test solutions were also added in the same quantities to a blank tube (reference). Both tubes were incubated at 100°C

for 10 min. After this time the tubes were placed in a cold water bath for 5 min. An aqueous solution of EtOH (60% v/v, 1 cm^3) was added to each tube. The resulting solutions were then mixed thoroughly and each filtered through a plug of glass wool. The sample and reference filtrates were made up to 3 cm^3 with the aqueous solution of 60% EtOH in sample and reference u.v. curvettes respectively. The absorbance of the sample solution was then measured against the reference blank at 570 nm.

(ii) **Quantitative Fmoc Test**

The acylated resin (*ca.* 3 mg) was removed from the reaction column and transferred to a sintered glass filter tube and washed as in the quantitative ninhydrin colour test (i) above but omitting treatment with piperidine-DMF. The acylated resin was then dried in a stream of nitrogen, the weight determined accurately, and transferred to a volumetric flask (10 cm^3) which was made up to the mark with a solution of 20% piperidine-DMF. After standing for a few minutes the absorbance of the sample solution was measured against a reference of 20% piperidine-DMF from 390-190 nm in sample and reference u.v. curvettes respectively.

Qualitative Determination

Qualitative Ninhydrin Colour Test, Kaiser Test

The following solutions were prepared: ninhydrin (0.5 g) in EtOH (10 cm^3); phenol (40.0 g) in EtOH (10 cm^3); aqueous KCN (0.001 *M*, 2 cm^3) in pyridine (98 cm^3).

A few beads of the acylated resin (*ca.* 5) were removed from the reaction column and transferred to a sintered glass filter tube and washed with DMF (2 x 3 cm^3), DCM (2 x 3 cm^3) and ether (2 x 3 cm^3). The beads were dried in a stream of nitrogen and transferred to a small ignition tube. One drop of each of the three solutions (prepared as above) was then added to the tube and to a blank tube (reference). Both tubes were then placed in an oven at 100°C for 5 min. To judge for complete acylation the colour of the contents of the sample tube was then compared to the colour of the contents of the reference tube. Complete reaction was indicated by the respective colours being identical, any blue colouration in the sample tube being indicative of incomplete reaction.

General Procedure for Cleavage/Deprotection

On completion of each synthesis the peptidyl-resin was subjected to cleavage/deprotection, using the following general method. Any deviations are indicated in the appropriate sections.

A trial cleavage/deprotection was initially employed; a small portion of the peptidyl-resin (*ca.* 25 mg) was treated with a mixture of TFA (40 cm^3), ethanedithiol (EDT) (40 µl, 1.0% v/v) and phenol (0.13 g, 3.3% w/v) at RT and small aliquots (*ca.* 100 µl) taken at intervals to determine the optimum reaction period for cleavage and deprotection. Each sample was evaporated under reduced pressure and the residue taken up in water, washed with ether and then analysed by analytical RPHPLC. Having optimised the conditions, the bulk of the peptidyl-resin was divided into two batches and each treated for the determined period with the nitrogen-purged cleavage/deprotection mixture (the amounts of reagents being adjusted accordingly).

The work-up procedure was then employed similarly as for the trial cleavages. The resin was first removed by filtration and washed with TFA. The combined filtrate and washings were evaporated under reduced pressure and the residue partitioned between water and ether, the aqueous layer washed further with four equal portions of ether and the combined ether layers back-extracted once with an equal volume of water. The combined aqueous layers were then lyophilised, having first removed residual ether by evaporation under reduced pressure, to yield the crude peptide generally as a white, fluffy solid, which was analysed by RPHPLC. The isolated crude peptide was then subjected to purification usually by a combination of gel permeation chromatography followed by preparative RPHPLC.

Purification Procedures

(i) Gel Permeation Chromatography

In some cases the crude peptides were subjected to gel permeation chromatography. The crude peptide (not more than 10 mg at a time) was applied to the column in the minimum volume of the eluting solution (in some instances a few drops of glacial acetic acid were added to achieve dissolution). Fractions (5 cm^3) were collected every 10 min, examined by analytical RPHPLC, and selected ones were pooled and lyophilised to afford the purified peptide.

(ii) **Preparative RPHPLC**

Preparative RPHPLC was used as a final purification technique usually following gel permeation chromatography. In general the standard preparative gradient was employed (as already described), unless otherwise stated. A trial purification was initially performed (using *ca.* 2 mg of material) before scale-up (on not more than 10 mg of material). The peptide to be purified was added to the column dissolved in the minimum volume of an aqueous solution of CH_3CN (10%, v/v). In some instances a further few drops of neat CH_3CN were required to achieve complete solubility.

Having loaded the material the gradient was initiated, and fractions (4 cm^3) collected each minute were examined by analytical RPHPLC. Those fractions seen to give rise to a single symmetrical peak were combined and lyophilised to yield the purified material.

Characterisation And Evaluation Of Purity

Each product was characterised by a combination of analytical RPHPLC, t.l.c., amino acid analysis and F.A.B. mass spectrometry. As a determination of purity it was imperative that each compound gave rise to a single, symmetrical peak by analytical RPHPLC and a single spot by t.l.c.. Certain compounds were studied further by ^1H n.m.r., the results of which are considered in the discussion. A small sample of each pure peptide was also subjected to biological testing.

***Bioassay Procedure**[4]

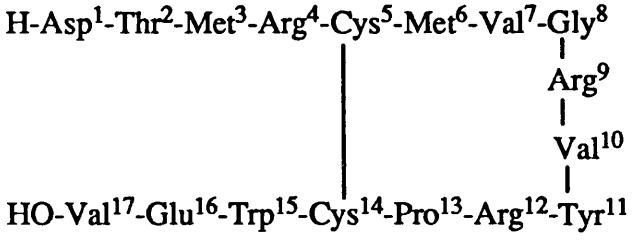
In early experiments, test peptides were weighed on an Oertling microbalance, dissolved in 1 m*M* HCl combining 0.1% bovine serum albumin (HPA) buffer, and stored at -40°C . In later experiments, peptides were dissolved in water-acetonitrile (1:1 v/v) and an aliquot taken for amino acid analysis. The remaining solution was dried under vacuum, and dissolved in an appropriate volume of HPA buffer as before.

A Chinese grass carp *Ctenophryngodon idellus* of 25-40 g was decapitated, the scales scraped off and placed into Hanks balanced salt solution combining 1% bovine serum albumin and 10^{-4} *M* phentolamine. The pH was adjusted to 7.5 with sodium bicarbonate. After the melanophores of the skin had fully dispersed, 2-3 scales were placed into each of the serial dilutions of the peptide solution to be assessed, prepared

with the same solution in a tissue-culture well-plate. The melanophore index was determined after 1 h. Synthetic MCH (Peninsula Laboratories) was used as a standard. The potency of each peptide was determined in two or three separate assays.*

Experimental to Chapter 2

Sequential Fragments of MCH:



MCH (**1**)

linear MCH(15-17) (**17**)
[Acm-Cys^{14}] linear MCH(14-17) (**18**)
[Acm-Cys^{14}] linear MCH(13-17) (**19**)
[Acm-Cys^{14}] linear MCH(12-17) (**20**)
[Acm-Cys^{14}] linear MCH(11-17) (**21**)
[Acm-Cys^{14}] linear MCH(10-17) (**22**)
[Acm-Cys^{14}] linear MCH(9-17) (**23**)
[Acm-Cys^{14}] linear MCH(8-17) (**24**)
[Acm-Cys^{14}] linear MCH(7-17) (**25**)
[Acm-Cys^{14}] linear MCH(6-17) (**26**)
[Acm-Cys^{5,14}] linear MCH(5-17) (**27**)
[Acm-Cys^{5,14}] linear MCH(4-17) (**28**)
[Acm-Cys^{5,14}] linear MCH(3-17) (**29**)
[Acm-Cys^{5,14}] linear MCH(2-17) (**30**)
[Acm-Cys^{5,14}] linear MCH (**31**)

A total of 2.5 g of resin (0.25 mequiv.) was used. For incorporation of the first Fmoc-amino acid, two sequential batches of Fmoc-Val-OPfp and DMAP were added with recirculation periods of 4 h and 15 h respectively. No quantitative ninhydrin colour test or Fmoc determination was employed for these early syntheses. The Fmoc group was cleaved and the synthesis was then continued following the usual synthetic cycle adding each Fmoc-amino acid derivative until the complete MCH sequence had been assembled. After each deprotection stage from the tripeptide onwards, a portion of the peptidyl-resin was removed and washed and dried in the usual manner, to make available the corresponding peptides, **(17)-(31)**. The following Fmoc-amino acids were coupled employing Pfp esters unless otherwise stated (the coupling times are indicated in parentheses): Fmoc-Glu(O-*t*-Bu) (25 min); Fmoc-Trp (25 min); Fmoc-Cys(Acm) (25 min); Fmoc-Pro (120 min); Fmoc-Arg(Mtr) (Dhbt ester, 25 min); Fmoc-Tyr(*t*-Bu) (25 min); Fmoc-Val (25 min); Fmoc-Arg(Mtr) (Dhbt ester, 100 min); Fmoc-Gly (25 min); Fmoc-Val (25 min); Fmoc-Met (25 min);

Fmoc-Cys(Acm) (120 min); Fmoc-Arg(Mtr) (Dhbt ester, 25 min); Fmoc-Met (25 min); Fmoc-Thr(*t*-Bu) (Dhbt ester, 25 min); Fmoc-Asp(O-*t*-Bu) (25 min).

Cleavage/deprotection of these peptidyl-resins gave samples of all the peptides in the series **(17)-(31)** with Acm protecting groups on cysteine residues where present. The general procedure for cleavage/deprotection was utilised in each case on 100 mg of the peptidyl-resin. The optimum period was found to be 7 h. However, a modified cleavage/deprotection regime was used for the peptidyl-resins of the title compounds **(17)**, **(18)** and **(19)**. In these cases the TFA contained anisole (3.6% v/v) and EDT (1% v/v), and the optimum cleavage/deprotection period was 2 h.

Purification of the crude peptides obtained after work-up was effected by semi-preparative RPHPLC in the usual fashion to afford the following *title compounds*:

(17) 0.8 mg (19%); **(18)** 1.1 mg (18%); **(19)** 1.2 mg (17%); **(20)** 2.2 mg (26%); **(21)** 2.4 mg (23%); **(22)** 2.4 mg (21%); **(23)** 2.7 mg (21%); **(24)** 2.8 mg (21%); **(25)** 3.0 mg (21%); **(26)** 2.9 mg (19%); **(27)** 3.1 mg (18%); **(28)** 3.1 mg (16%); **(29)** 3.2 mg (16%); **(30)** 3.0 mg (14%); **(31)** 3.1 mg (14%).

Each compound was characterised as follows:

(17) analytical RPHPLC (apparatus A) 51% B, t_R 28.0 min; t.l.c. R_f 0.34 (S1), R_f 0.61 (S2), R_f 0.83 (S3); amino acid analysis: Glu 1.06, Val 1.00; m/z (F.A.B., glycerol matrix) 433 $(M+H)^+$ [$(M+H)^+$ calcd. 433.2].

(18) analytical RPHPLC (apparatus A) 50% B, t_R 27.5 min; t.l.c. R_f 0.33 (S1), R_f 0.61 (S2), R_f 0.82 (S3); amino acid analysis: Glu 1.08, Val 1.00; m/z (F.A.B., glycerol matrix) 607 $(M+H)^+$ [$(M+H)^+$ calcd. 607.3].

(19) analytical RPHPLC (apparatus A) 50% B, t_R 27.5 min; t.l.c. R_f 0.34 (S1), R_f 0.60 (S2), R_f 0.83 (S3); amino acid analysis: Glu 1.04, Pro 0.95, Val 1.00; m/z (F.A.B., thioglycerol matrix) 704 $(M+H)^+$ [$(M+H)^+$ calcd. 704.3].

(20) analytical RPHPLC (apparatus A) 51% B, t_R 28.0 min; t.l.c. R_f 0.35 (S1), R_f 0.59 (S2), R_f 0.71 (S3); amino acid analysis: Glu 1.05, Arg 0.92, Pro 1.05, Val 1.00; m/z (F.A.B., glycerol matrix) 860 $(M+H)^+$ [$(M+H)^+$ calcd. 860.4].

(21) analytical RPHPLC (apparatus A) 53% B, t_R 29.0 min; t.l.c. R_f 0.39 (S1), R_f 0.62 (S2), R_f 0.72 (S3); amino acid analysis: Glu 1.07, Arg 1.05, Pro 1.06, Tyr 0.90, Val 1.00; m/z (F.A.B., glycerol matrix) 1023 $(M+H)^+$ [$(M+H)^+$ calcd. 1023.5].

(22) analytical RPHPLC (apparatus A) 53% B, t_R 29.0 min; t.l.c. R_f 0.38 (S1), R_f 0.60 (S2), R_f 0.73 (S3); amino acid analysis: Glu 1.05, Arg 1.04, Pro 0.96, Tyr 0.92, Val 2.00; m/z (F.A.B., glycerol matrix) 1123 $(M+H)^+$ [$(M+H)^+$ calcd. 1122.6].

(23) analytical RPHPLC (apparatus A) 54% B, t_R 29.5 min; t.l.c. R_f 0.40 (S1), R_f 0.64 (S2), R_f 0.77 (S3); amino acid analysis: Glu 1.03, Arg 2.16, Pro 1.02, Tyr 0.89, Val 2.00; m/z (F.A.B., thioglycerol matrix) 1279 $(M+H)^+$ [$(M+H)^+$ calcd. 1278.7].

(24) analytical RPHPLC (apparatus A) 53% B, t_R 29.0 min; t.l.c. R_f 0.40 (S1), R_f 0.62 (S2), R_f 0.75 (S3); amino acid analysis: Glu 1.07, Gly 1.05, Arg 2.13, Pro 0.94, Tyr 0.90, Val 2.00; m/z (F.A.B., thioglycerol matrix) 1336 $(M+H)^+$ [$(M+H)^+$ calcd. 1335.7].

(25) analytical RPHPLC (apparatus A) 54% B, t_R 29.5 min; t.l.c. R_f 0.40 (S1), R_f 0.66 (S2), R_f 0.78 (S3); amino acid analysis: Glu 1.08, Gly 1.02, Arg 2.07, Pro 0.98, Tyr 0.90, Val 3.00; m/z (F.A.B., thioglycerol matrix) 1435 $(M+H)^+$ [$(M+H)^+$ calcd. 1434.8].

(26) analytical RPHPLC (apparatus A) 55% B, t_R 30.0 min; t.l.c. R_f 0.42 (S1), R_f 0.68 (S2), R_f 0.78 (S3); amino acid analysis: Glu 1.02, Gly 1.08, Arg 2.03, Pro 1.01, Tyr 0.95, Val 3.00, Met 0.88; m/z (F.A.B.,thioglycerol matrix) 1566 $(M+H)^+$ [$(M+H)^+$ calcd. 1565.8].

(27) analytical RPHPLC (apparatus A) 57% B, t_R 31.0 min; t.l.c. R_f 0.44 (S1), R_f 0.69 (S2), R_f 0.80 (S3); amino acid analysis: Glu 1.05, Gly 0.93, Arg 1.98, Pro 0.98, Tyr 0.89, Val 3.00, Met 0.89; m/z (F.A.B., thioglycerol matrix) 1740 $(M+H)^+$ [$(M+H)^+$ calcd. 1739.9].

(28) analytical RPHPLC (apparatus A) 57% B, t_R 31.0 min; t.l.c. R_f 0.44 (S1), R_f 0.68 (S2), R_f 0.81 (S3); amino acid analysis: Glu 0.98, Gly 0.94, Arg 3.04, Pro 0.94, Tyr 0.93, Val 3.00, Met 0.90; m/z (F.A.B., thioglycerol matrix) 1896 $(M+H)^+$ [$(M+H)^+$ calcd. 1896.0].

(29) analytical RPHPLC (apparatus A) 58% B, t_R 31.5 min; t.l.c. R_f 0.44 (S1), R_f 0.67 (S2), R_f 0.83 (S3); amino acid analysis: Glu 1.06, Gly 0.98, Arg 3.03, Pro 1.01, Tyr 0.91, Val 3.00, Met 1.94; m/z (F.A.B., glycerol matrix) 2027 $(M+H)^+$ [$(M+H)^+$ calcd. 2027.0].

(30) analytical RPHPLC (apparatus A) 58% B, 31.5 min; t.l.c. R_f 0.63 (S1), R_f 0.69 (S2), R_f 0.81 (S3); amino acid analysis: Glu 0.99, Gly 1.01, Arg 3.09, Thr 0.99, Pro 1.02, Tyr 0.90, Val 3.00, Met 1.89; m/z (F.A.B., glycerol matrix) 2128 $(M+H)^+$ [$(M+H)^+$ calcd. 2128.1].

(31) analytical RPHPLC (apparatus A) 58% B, t_R 31.5 min; t.l.c. R_f 0.63 (S1), R_f 0.69 (S2), R_f 0.80 (S3); amino acid analysis: Asp 1.01, Glu 1.10, Gly 1.07, Arg 3.03, Thr 1.05, Pro 1.07, Tyr 0.89, Val 3.00, Met 1.89; m/z (F.A.B., thioglycerol matrix) 2243 $(M+H)^+$ [$(M+H)^+$ calcd. 2243.1].

[Acm-Cys^{5,14}, Phe^{11}] linear MCH (32).- In a parallel synthesis with that described above the Phe analogue (32) was prepared, adding Fmoc-Phe-OPfp with a coupling time of 50 min, in place of Fmoc-Tyr-OPfp.

The general procedure for cleavage/deprotection was adhered to on 100 mg of peptidyl-resin with an optimum period of 7 h. In this case analytical RPHPLC was performed on a 10 μ C_{18} Vydac column (250 mm x 4.6 mm diameter) with all other conditions as normal. Purification was carried out by preparative RPHPLC alone under different conditions to those normally used. Chromatography was performed on a 10 μ C_{18} Vydac column (250 mm x 10 mm diameter) using the standard profile, at a flow rate of 3 cm^3 per min. The crude peptide was loaded in the minimum volume of 5% B and fractions (3 cm^3) were collected each minute. All other conditions were as normal. This gave the purified *title compound* (32) (3.2 mg, 14%); analytical RPHPLC (apparatus B, 10 μ C_{18} Vydac column) 43% B, t_R 24.0 min; t.l.c. R_f 0.62 (S1), R_f 0.70 (S2), R_f 0.82 (S3); amino acid analysis: Asp 1.02, Glu 0.98, Gly 0.97, Arg 2.97, Thr 1.01, Pro 1.01, Val 3.00, Met 1.80, Phe 0.96; m/z (F.A.B., glycerol matrix) 2227 $(M+H)^+$ [$(M+H)^+$ calcd. 2227.1].

Linear MCH(1-4,15-17)(35).- A total of 1.19 g of resin (0.1 mequiv.) was utilised. Two sequential batches of the freshly prepared symmetrical anhydride of Fmoc-Val were added with DMAP and each recirculated for 50 min. The quantitative ninhydrin colour test indicated that 91% esterification had been achieved. After removal of the Fmoc group the cycle for the incorporation of the remaining Fmoc-amino acids was

employed using Pfp esters unless stated otherwise. The following Fmoc-amino acids were coupled: Fmoc-Glu(O-*t*-Bu) (25 min); Fmoc-Trp (25 min). On removal of the Fmoc group the symmetrical anhydride of Fmoc-Arg(Mtr) was prepared and added immediately in the usual fashion. After a recirculation time of 50 min, the Kaiser test indicated that acylation was incomplete. A fresh batch of anhydride was added and, following a recirculation time of 50 min, a satisfactory Kaiser test result was obtained. The synthesis then proceeded in the usual fashion, following cleavage of the Fmoc group. The following Fmoc-amino acids were coupled: Fmoc-Met (25 min); Fmoc-Thr(*t*-Bu) (Dhbt ester, 25 min); Fmoc-Asp(O-*t*-Bu) (25 min).

Using the usual conditions the cleavage/deprotection of 500 mg of peptidyl-resin was allowed to proceed for an optimum period of 7 h. All analytical RPHPLC was performed using a profile of 5 min at 25% followed by a linear gradient of 2% per min to 90% B, all other conditions being as standard. Purification was effected by a combination of gel permeation chromatography (Bio-Gel P-2) and preparative RPHPLC using a profile of 5 min at 30% B followed by a linear gradient of 1% per min to 70% B (other conditions were as usual) loading the peptide as a solution in 30% B. This furnished the *title compound* (**35**) (15.4 mg, 33%); analytical RPHPLC (apparatus A, profile of 5 min at 25% B followed by a linear gradient of 2% per min to 90% B) 48% B, t_R 16.5 min; t.l.c. R_f 0.23 (S1), R_f 0.36 (S2); amino acid analysis: Asp 1.11, Glu 1.01, Arg 1.10, Thr 1.01, Val 1.00, Met 0.88; m/z (F.A.B., glycerol matrix) 936 $(M+H)^+$ [$(M+H)^+$ calcd. 936.5].

Linear MCH (1-4,Aha, 15-17) (**36**)

(a) *Preparation of Pentafluorophenyl* N^{α}-*(9-fluorenylmethyloxycarbonyl)-7-aminoheptanoate* (**37**)

(i) *7-Aminoheptanoic acid hydrochloride* (**38**).- 2-Azacyclooctanone (2.00 g, 15.7 mmol) was dissolved in 2 *N* hydrochloric acid (100 cm^3) and heated under reflux for 1.5 h. The solution was then evaporated under reduced pressure and the residue triturated with ether to afford *7-aminoheptanoic acid hydrochloride* (**38**) (2.78 g, 98%) as a pale brown solid which was used without further purification. A small portion was purified for analytical purposes by recrystallisation from acetone to give a clear crystalline solid, m.p. 105-106°C; υ_{max} (Nujol) 3200-2700 (COOH and NH_3^+), 2000 (NH_3^+), 1710 (COOH), 1610 (NH_3^+), 1440 cm^{-1}; δ_H (DMSO-d_6) 1.29-1.57 (8H, c, $(CH_2)_4$), 2.21 (2H, t, J = 7.3Hz, $C\underline{H}_2COOH$), 2.73 (2H, c, $C\underline{H}_2NH_3^+$), 8.15 (4H, bs, NH_3^+ and COOH); δ_C (DMSO-d_6) 24.2, 25.5, 26.6, 27.9 ($(CH_2)_4$),

33.4 ($\underline{C}H_2COOH$), 38.5 ($CH_2NH_3^+$), 174.3 (COOH); m/z (C.I., NH_3) no $(M+H)^+$, 146 $(M-Cl)^+$ (100%), 128 (5); [Found: C, 46.6; H, 9.1; N, 7.8. $C_7H_{16}O_2NCl$ requires C, 46.6; H, 8.9; N, 7.7%].

(ii) N^{α}-*(9-Fluorenylmethyloxycarbonyl)-7-aminoheptonoic acid*[77] **(39)**.- 7-Aminoheptanoic acid hydrochloride **(38)** (2.56 g, 14.1 mmol) was suspended with stirring in aqueous Na_2CO_3 (10% w/v, 70 cm^3) and cooled in an ice-bath. Dioxan (14 cm^3) was added followed by the slow addition of a solution of 9-fluorenylmethyl chloroformate (3.70 g, 14.3 mmol) in dioxan (22 cm^3). The mixture was stirred at 0°C for 1 h and then at RT for 18 h during which time further white solid developed in the suspension. The mixture was concentrated to low bulk under reduced pressure and was added to cold aqueous Na_2CO_3 (10% w/v, 200 cm^3) and then washed with ether (3 x 50 cm^3). The aqueous layer was acidified with concentrated hydrochloric acid to pH 2 to yield a white crystalline solid which was collected by filtration, washed with 0.1 *N* hydrochloric acid (2 x 20 cm^3) and water (2 x 30 cm^3) and air dried. Purification was effected by column chromatography [silica gel, $CHCl_3$ - EtOH (8:1)] and afforded N^{α}-*(9-fluorenylmethyloxycarbonyl)-7-aminoheptanoic acid* **(39)** (3.21 g, 62%) as a white solid, m.p. 149-151°C, t.l.c. R_f 0.64 ($CHCl_3$-EtOH 7:1 v/v); υ_{max} (Nujol) 3320 (NH), 3100-2600 (COOH), 1710 (COOH), 1680 (O-CO-NH), 1520 (CO-NH), 1440 cm^{-1}; δ_H (DMSO-d_6) 1.25-1.49 (8H, c, $(CH_2)_4$), 2.20 (2H, t, J = 7.3Hz, $C\underline{H}_2COOH$), 2.96 (2H, dt, J = 7.2, 6.2Hz, $C\underline{H}_2NH$), 3.35 (1H, bs, NH), 4.29 (3H, m, Fmoc $CHCH_2$), 7.31, 7.41, 7.67, 7.87 (2H, d, J = 7.5Hz; 2H, d, J = 7.3Hz; 2H, dd, J = 7.0, 7.3Hz; 2H, dd, J = 7.3, 7.5Hz, Fmoc H-aromatic); δ_C (DMSO-d_6) 24.5, 25.9, 28.3, 29.2 ($(CH_2)_4$), 33.8 ($\underline{C}H_2COOH$), 40.1 (CH_2NH), 46.7 (Fmoc $\underline{C}HCH_2$), 65.1 (Fmoc $CH\underline{C}H_2$), 120.0, 125.1, 127.0, 127.5, 140.7, 143.9 (Fmoc C-aromatic), 156.4 (Fmoc C=O), 174.5 (COOH); m/z (C.I., NH_3) 368 $(M+H)^+$ (2%), 214 (50), 179 (72), 146 (100); [Found: C, 71.8; H, 6.6; N, 3.5. $C_{22}H_{25}NO_4$ requires C, 71.9; H, 6.9; N, 3.8%].

(iii) *Pentafluorophenyl* N^{α}-*(9-fluorenylmethyloxycarbonyl)-7-aminoheptanoate*[79] **(37)**.- N^{α}-(9-fluorenylmethyloxycarbonyl)-7-aminoheptanoic acid **(39)** (1.04 g, 2.8 mmol) and pentafluorophenol (0.50 g, 2.7 mmol) were dissolved in a stirred mixture of DMF-EtOAc (1:1 v/v, 12 cm^3) at 0°C while DCC (0.58 g, 2.8 mmol) was added portionwise. The solution was stirred for 1 h at 0°C and for 1 h at R.T. The precipitated DCU was then removed by filtration and the filtrate concentrated under reduced pressure. The residue was triturated

with *n*-hexane and filtered to afford the *title compound* (**37**) (1.21 g, 80%) as a white solid, m.p. 99-101°C (recrystallised petrol), t.l.c. R_f 0.57 (petrol-EtOAc 4:1 v/v). (Quantitative comparison with a sample of Fmoc-Leu-OPfp by ^{19}F n.m.r. showed the pentafluorophenyl group to be present as required); υ_{max} (Nujol) 3340 (NH), 1780 (CO-O), 1680 (O-CO-NH), 1510 (NH), 1440 cm^{-1}; δ_H (DMSO-d_6) 1.32-1.66 (8H, c, $(CH_2)_4$), 2.77 (2H, t, J = 7.3 Hz, CH_2-CO-OPfp), 2.98 (2H, dt, J = 7.2, 6.2Hz, $C\underline{H}_2NH$), 3.35 (1H, bs, NH), 4.26 (3H, m, Fmoc $CHCH_2$), 7.33, 7.41, 7.69, 7.89 (2H, d, J = 7.5 Hz; 2H, d, J = 7.3 Hz; 2H, dd, J = 7.0, 7.3 Hz; 2H, dd, J = 7.3, 7.5 Hz, Fmoc H-aromatic); δ_C (DMSO-d_6) 24.2, 25.7, 27.8, 29.1 ($(CH_2)_4$), 32.4 ($\underline{C}H_2$-CO-OPfp), 40.1 (CH_2NH), 46.7 (Fmoc $\underline{C}HCH_2$), 65.1 (Fmoc $CH\underline{C}H_2$), 120.0, 125.1, 127.0, 127.5, 140.7, 143.9 (Fmoc C-aromatic), 156.0 (Fmoc C=O), 169.5 ($\underline{C}$O-OPfp); m/z (C.I.) 534 $(M+1)^+$ (53%), 350 (24), 312 (77), 265 (50), 225 $(CH_2$-CO-OPfp$)^+$ (67), 178 (100); [Found: C, 62.7; H, 4.6; N, 2.7. $C_{28}H_{24}NO_4F_5$ requires C, 63.0; H, 4.5; N, 2.6%].

(b) *Incorporation of 7-Aminoheptanoic acid in the synthesis of* (**36**)

In a parallel synthesis to that of (**35**), Fmoc-Aha-OPfp was incorporated into the growing peptide chain in the usual manner. Although acylation was complete after the normal 25 min period, the removal of the Fmoc group was observed to be exceptionally slow and exposure to piperidine-DMF was continued for 15 h. All subsequent acylations and deprotections were as normal.

The usual cleavage/deprotection procedure was adhered to, for 500 mg of peptidyl-resin, with an optimum period of 7 h. On work-up the crude peptide was subjected to an initial purification by gel permeation chromatography (Bio-Gel P-2). Preparative RPHPLC was then performed, using identical conditions to those employed for the purification of (**35**), though a few drops of neat CH_3CN were required to achieve dissolution of the peptide in the loading medium. All analytical RPHPLC was also carried out under the same conditions used for (**35**). The purified *title compound* (**36**) (20.7 mg, 39%) was furnished; analytical RPHPLC (apparatus A, profile of 5 min at 25% B followed by a linear gradient of 2% per min to 90% B) 50% B, t_R 17.5 min; t.l.c. R_f 0.28 (S1), R_f 0.42 (S2); amino acid analysis: Asp 0.97, Glu 1.10, Arg 1.08, Thr 1.09, Val 1.00, Met 0.85; m/z (F.A.B., glycerol matrix) 1064 $(M+H)^+$ [$(M+H)^+$ calcd. 1063.6].

[Acm-Cys^{5,14}] linear MCH(5-14) (**43**).- A total of 1.1 g of resin (0.1 mequiv.) was used. Two batches of Fmoc-Cys(Acm)-OPfp were employed, each with recirculation periods of 4 h, using 0.025 molar equivalents of DMAP. The quantitative ninhydrin colour test indicated that esterification had proceeded to an extent of 85%. The Fmoc group was cleaved and the synthesis carried out conforming to the usual protocol. The following Fmoc-amino acids were coupled employing Pfp esters throughout unless stated otherwise: Fmoc-Pro (120 min); Fmoc-Arg(Mtr) (Dhbt ester, 25 min); Fmoc-Tyr(*t*-Bu) (25 min); Fmoc-Val (25 min); Fmoc-Arg(Mtr) (Dhbt ester, 50 min); Fmoc-Gly (25 min); Fmoc-Val (25 min); Fmoc-Met (25 min); Fmoc-Cys(Acm) (25 min).

Cleavage/deprotection proceeded as usual on 1.0 g of peptidyl-resin with an optimum period of 15 h. Following work-up purification was achieved by gel permeation chromatography (Bio-Gel P-2) followed by preparative RPHPLC under the customary conditions to furnish the *title compound* (**43**) (91.4 mg, 69%); analytical RPHPLC (apparatus A) 49% B, t_R 27.0 min; t.l.c. R_f 0.30 (S1), R_f 0.81 (S3); amino acid analysis: Gly 1.03, Arg 2.05, Pro 1.01, Tyr 0.89, Val 2.00, Met 0.96; m/z (F.A.B., thioglycerol matrix) 1326 $(M+H)^+$ [$(M+H)^+$ calcd. 1325.7].

[Acm-Cys^{5,14}, Phe^{11}) linear MCH(5-14) (**45**).- In a parallel synthesis to that of (**43**), the Phe analogue was prepared by the addition of Fmoc-Phe-OPfp, substituted for Fmoc-Tyr(*t*-Bu)-OPfp with a coupling time of 25 min. The cleavage/deprotection on 500 mg of peptidyl-resin proceeded in the usual fashion with an optimum period of 15 h. The crude peptide was purified by a combination of gel permeation chromatography (Bio-Gel P-2) and preparative RPHPLC to render the *title compound* (**45**) (42.3 mg, 65%); analytical RPHPLC (apparatus A) 53% B, t_R 29.0 min; t.l.c. R_f 0.35 (S1), R_f 0.83 (S3); amino acid analysis: Gly 0.99, Arg 1.89, Pro 0.94, Val 2.00, Met 0.88, Phe 0.97; m/z (F.A.B., thioglycerol matrix) 1310 $(M+H)^+$ [$(M+H)^+$ calcd. 1309.7].

Cyclisation Reactions

In most cases a quantitative thiol test was employed, based on the Ellman procedure[73], as a monitor of the cyclisation reaction. By this method it was possible to detect and quantify any free thiol present.

Quantitative Thiol Test Procedure

The Ellman stock solution was prepared by dissolving 5,5′-dithio*bis*-(2-nitrobenzoic acid) (3.0 mg, 7.6 μmol) in 100 cm^3 of 10 m*M* aqueous ammonium bicarbonate solution. A solution of cysteine hydrochloride (1.6 mg, 10.0 μmol) in distilled water (5 cm^3) was also prepared as a standard for comparison with the sample to be estimated. The stock solution (2.5 cm^3) was added to a u.v. curvette, which was placed in a constant wavelength detector at 450 nm and the absorbance adjusted to zero. The following quantities of the standard cysteine solution were added to the curvette and the absorbance measured at 450 nm in each case: 5 μl, 10 μl, 20 μl, 40 μl. An aqueous solution of the sample to be tested was then prepared (*ca.* 0.4 μmol of sample in 100 μl of distilled water). A portion (10 μl) of the sample solution was added to 2.5 cm^3 of fresh stock solution in the curvette and the absorbance determined at 450 nm. Alternatively the appropriate volume was removed from the oxidation solution during aeration processes and added directly to the curvette. The development of a yellow colour on addition of the sample solution to the stock solution was an indication of the presence of free thiol in the sample.

Cyclic MCH(5-14) (**7**) - From (1) [Acm-$Cys^{5,14}$] linear MCH(5-14) (**43**)

(i) **Iodine Mediated Oxidation**

The MCH derivative (**43**) (10.0 mg, 7.5 μmol) was dissolved in an aqueous solution of 90% acetic acid (v/v, 1.5 cm^3) containing 2 *M* hydrochloric acid (10 μl). The resulting mixture was added dropwise over 4 min to a stirred solution of iodine (195 mg, 0.8 mmol) in 90% acetic acid (15 cm^3). Stirring was continued for a further 30 min at RT and the mixture was poured into cold water (150 cm^3) and the unreacted iodine extracted with carbon tetrachloride (3 x 30 cm^3). The combined organic layers were back-extracted with water (3 x 30 cm^3). The combined aqueous layers were then lyophilised to afford a yellow residue which was dissolved in an aqueous solution of 90% acetic acid (2 cm^3) and concentrated aqueous ascorbic acid solution was then added until a colourless mixture resulted. This was then added directly to a gel permeation column (Bio-Gel P-2) run under the usual conditions. The peptidic material yielded was further purified by preparative RPHPLC to afford only 0.5 mg (6%) of material which was shown by F.A.B. mass spectrometry to correspond to [O-Met^6] cyclic MCH(5-14) (**47**). It was later found that the undesired oxidation of the methionine residue could be suppressed by performing the reaction under

nitrogen, in purged solutions, with the addition of methionine. However, isolation of the product from this system proceeded with difficulty and recovered yields of pure product never exceeded 6%.

(ii) **Treatment with Mercuric Acetate Followed by (a) Air Mediated Oxidation (b) Ferricyanide Mediated Oxidation**

An ammonium acetate buffer was prepared at pH 4.0 using aqueous acetic acid and aqueous ammonia solution. The compound **(43)** (33.1 mg, 25 μmol) was dissolved in the buffer (7 cm^3) under nitrogen with stirring. A solution of mercuric acetate (31.0 mg, 100 μmol) in the buffer (1 cm^3) was then added and the mixture stirred for 4 h at RT. Hydrogen sulphide was then bubbled through the mixture for 30 min followed by a nitrogen purge for 15 min. The precipitated HgS was removed by centrifugation in a sealed nitrogen purged centrifuge tube. The supernatant solution was concentrated to *ca.* 2 cm^3 under reduced pressure and a mixture of 2-mercaptoethanol (1 cm^3) and 4 *M* acetic acid (1 cm^3) added and the resultant solution stirred for 15 h under nitrogen. The mixture was then added directly to a gel permeation column (Bio-Gel P-2, 140 cm x 3.5 cm diameter) packed in and eluted with a nitrogen purged solution of 4 *M* acetic acid containing 10 m*M* mercaptoethanol. The effluent was monitored at the usual wavelength at a flow rate of 0.5 cm^3 per min; fractions (5 cm^3) being collected every 10 min. This afforded 13.0 mg (44%) of the crude dihydro peptide (linear MCH(5-14) **(48)**) which was used in the following cyclisation reactions without further purification.

A small portion (3.0 mg) was subjected to preparative RPHPLC to afford 2.1 mg of material which gave rise to a single symmetrical peak on analytical RPHPLC at 49% B, t_R 27.5 min using the standard gradient (apparatus A). A quantitative thiol test indicated that free sulphydryl was present (61% of free SH).

(a) **Air Mediated Oxidation**

A buffer was prepared from 0.5 *M* acetic acid with the pH adjusted to 7.0 using ammonium hydroxide solution. The crude linear dihydro peptide (linear MCH(5-14 **(48)**) (10.2 mg, 8.5 μmol) was dissolved in the nitrogen purged buffer (10 cm^3) in a 10 cm^3 conical flask (1 mg per cm^3 dilution). The solution was allowed to stand open to the atmosphere for 15 h

at RT during which time a small amount of precipitate developed. The thiol test confirmed the absence of free sulphydryls, the solution was lyophilised, and the crude material taken up in 2 *M* acetic acid (1 cm^3) and applied to a gel permeation column (Bio-Gel P-2, 140 cm x 3.5 cm diameter) packed in and eluted with 2 *M* acetic acid. The effluent was monitored at the usual wavelength, at a flow rate of 0.5 cm^3 per minute and fractions (5 cm^3) were collected every 10 min. This yielded 5.7 mg of material which was further purified by preparative RPHPLC to render the *title compound* (7) (2.1 mg, 20%); analytical RPHPLC (apparatus A) 49% B, t_R 27.0 min; t.l.c. R_f 0.29 (S1), R_f 0.58 (S2), R_f 0.80 (S3); amino acid analysis: Gly 1.08, Arg 2.10, Pro 1.08, Tyr 0.87, Val 2.00, Met 0.90; m/z (F.A.B., glycerol/diglycerol matrix) 1181 $(M+H)^+$ [$(M+H)^+$ calcd. 1181.5].

(b) **Ferricyanide Mediated Oxidation**

The linear dihydro peptide linear MCH(5-14) **(48)** (4.5 mg, 3.8 µmol) was dissolved in an aqueous solution of ammonium acetate buffer at pH 7.0 under nitrogen with stirring. An aqueous solution of potassium ferricyanide (10 m*M*) (420 µl, 4.2 µmol) was added dropwise and a permanent yellow colouration resulted. The solution was stirred for 2 h at RT and was then evaporated to dryness under reduced pressure. The residue was dissolved in 2 *M* acetic acid (2 cm^3) and applied to a gel permeation column (Bio-Gel P-2) using identical conditons to those employed for the purification of the product from the air oxidation process (a). Further purification by RPHPLC rendered 1.0 mg (22%) of product shown to be identical to that furnished by the air oxidation process (a). Complete absence of free sulphydryls was confirmed by the thiol test.

Cyclic MCH(5-14) (7) - From (2) [Trt-$Cys^{5,14}$]-peptidyl-resin

(i) **Preparation of the Peptidyl-resin**

The synthesis was performed in the usual fashion using 2.22 g of resin (0.2 mequiv.). For incorporation of the first Fmoc-amino acid, Fmoc-Cys(Trt)-OPfp and DMAP (0.025 molar equivalents) were added with a recirculation time of 2 h. Two further batches were then employed with recirculation periods of 15 h and 2 h respectively. The quantitative ninhydrin colour test indicated a resin coverage of 74%. The Fmoc group was then cleaved and the following Fmoc-amino acids were coupled, using Pfp esters

unless stated otherwise: Fmoc-Pro (120 min); Fmoc-Arg(Mtr) (Dhbt ester, 25 min); Fmoc-Tyr(*t*-Bu) (25 min); Fmoc-Val (60 min); Fmoc-Arg(Mtr) (Dhbt ester, 60 min); Fmoc-Gly (25 min); Fmoc-Val (90 min); Fmoc-Met (25 min); Fmoc-Cys(Trt) (25 min).

(ii) **Cyclisation Procedure**

The peptidyl-resin (1.00 g) was added to the usual cleavage/deprotection mixture and a yellow colour instantly developed in solution. The mixture was left to stand for 15 h, then filtered and the resin washed with TFA. In contrast to the normal work-up procedure the filtrate and washings were reduced in volume to about one sixth of the original and were then poured into 10-fold the volume of water. The solution was then washed as usual using *t*-butyl methyl ether and lyophilisation of the combined aqueous layers yielded 50.1 mg of the crude linear disulphydryl compound linear MCH(5-14) (**48**). The quantitative thiol test indicated the presence of free thiol groups (70%). The crude peptide was added without delay to a nitrogen purged solution of ammonium acetate buffer (50 cm^3) at pH 7.5 in a 50 cm^3 conical flask (*ca.* 1 mg per cm^3). The mixture was left to stand open to the air for 15 h at RT. The thiol test confirmed that no free sulphydryls were present. The solution was lyophilised and the crude material applied to a gel permeation column (Bio-Gel P-2) using identical conditions to those employed in (1) (ii) (a) to afford 41.4 mg of material which was further purified by preparative RPHPLC to yield the *title compound* (**7**) (33.2 mg, 28%). The product was characterised identically to the compound yielded from method (1) (ii) (a).

Cyclic MCH (5-17) (**5**)

(i) **Preparation of the peptidyl-resin**

The synthesis was performed as usual on 2.00 g of resin (0.2 mequiv.). The symmetrical anhydride of Fmoc-Val was first prepared and added with DMAP employing a recirculation period of 50 min before a second batch was added and recirculated similarly. The quantitative ninhydrin colour test indicated a resin coverage of 77% had been attained. The Fmoc group was cleaved and the synthesis proceeded using Pfp esters unless otherwise stated. The following Fmoc-amino acids were coupled: Fmoc-Glu(O-*t*-Bu) (25 min); Fmoc-Trp (25 min); Fmoc-Cys(Trt) (25 min);

Fmoc-Pro (120 min); Fmoc-Arg(Mtr) (Dhbt ester, 25 min); Fmoc-Tyr(*t*-Bu) (25 min); Fmoc-Val (25 min); Fmoc-Arg(Mtr) (Dhbt ester, 60 min); Fmoc-Gly (25 min); Fmoc-Val (25 min); Fmoc-Met (25 min); Fmoc-Cys(Trt) (90 min).

(ii) **Cyclisation Procedure**

The peptidyl-resin (500 mg) was subjected to the usual cleavage/deprotection mixture, for 7 h. An identical work-up procedure as that used for cyclic MCH(5-14) (**7**) in method (2) (ii) was employed. However, air oxidation was allowed to proceed before lyophilisation of the aqueous layers. After the ether wash the combined aqueous layers were placed in a suitable narrow necked conical flask and the total volume adjusted by the addition of water so as to give an estimated concentration of *ca.* 1 mg of peptide per cm^3. The pH of the solution was adjusted to 7.6 by the addition of ammonium hydroxide solution whilst degassing with a nitrogen purge. The solution was left to stand for 30 h before lyophilisation. This afforded the crude peptide in the presence of contaminating ammonium trifluoroacetate salt. The peptide was isolated by **solid phase extraction.** This was achieved by passage through a small cartridge of C_{18} silica (8 mm x 5 mm diameter), connected to a mini pump, at a flow rate of *ca.* 2 cm^3 per min. The initially dry packing material was pretreated by pumping through an aqueous solution of 70% CH_3CN (10 cm^3) followed by 5% CH_3CN (10 cm^3). A portion (200 mg) of the peptide/salt material was then suspended in 5% CH_3CN containing a drop of TFA (30 cm^3). The suspension was pumped through the column whilst collecting the effluent, which was then recirculated through the column for 20 minutes. Water (20 cm^3) followed by 5% CH_3CN (20 cm^3) were then passed successively through the column to wash away any remaining salt and other contaminants. Finally the concentration of CH_3CN was increased to 70% to elute the peptide to a saved vessel. After pumping 70% CH_3CN for a further 10 min, the effluent was lyophilised to yield the crude peptide now free of the contaminating salt. The procedure was then repeated for the remainder of the peptide/salt material to afford a total of 22.1 mg of crude peptide. The thiol test indicated the absence of free sulphydryls.

The crude peptide was further purified by gel permeation chromatography on a column of Bio-Gel P-2 (140 cm x 3 cm diameter) packed in and eluted with 2 *M* acetic acid at a flow rate of 0.5 cm^3 per min.

Glacial acetic acid was added dropwise to achieve dissolution of the crude peptide in the loading medium. Fractions (5 cm^3) were collected every 10 min with other conditions as usual. This afforded the *title compound* (**5**) (22.4 mg, 28%). A portion (7.1 mg) was subjected to purification by preparative RPHPLC to render 3.0 mg of material (a higher concentration of CH_3CN, *ca.* 70%, was required to dissolve the peptide for loading); analytical RPHPLC (apparatus A) 55% B, t_R 30.0 min; t.l.c. R_f 0.41 (S1), R_f 0.66 (S2), R_f 0.79 (S3); amino acid analysis: Glu 1.01, Gly 1.09, Arg 2.01, Pro 1.06, Tyr 0.93, Val 3.00, Met 0.89; m/z (F.A.B., glycerol/diglycerol matrix) 1596 $(M+H)^+$ [$(M+H)^+$ calcd. 1595.8].

MCH (**1**)

(i) **Preparation of the peptidyl-resin**

In a parallel synthesis to that of cyclic MCH(5-17) (**5**) the assembly was continued until the complete MCH sequence had been assembled. The following Fmoc-amino acid derivatives were added: Fmoc-Arg(Mtr)-ODhbt (25 min); Fmoc-Met-OPfp (25 min); Fmoc-Thr(*t*-Bu)-ODhbt (25 min); Fmoc-Asp(O-*t*-Bu)-OPfp (25 min).

(ii) **Cyclisation Procedure**

The peptidyl-resin (500 mg) was added to the usual cleavage/deprotection mixture for 7 h. The work-up, cyclisation procedure and subsequent removal of ammonium trifluoroacetate salt proceeded as for (**5**). The crude peptide, which was shown to lack any free sulphydryls, was purified by gel permeation chromatography (Sephadex LH20) under the usual conditions to afford 30.1 mg. Preparative RPHPLC was carried out on a 10 μ C_{18} Vydac column (250 mm x 10 mm diameter) using the standard gradient, at a flow rate of 3 cm^3 per minute. The peptide was loaded in the minimum volume of 5% B and fractions (3 cm^3) were collected each minute, to afford the *title compound* (**1**) (14.4 mg, 13%). The product was shown to give rise to a single peak on analytical RPHPLC corresponding exactly to that of a sample of synthetic MCH (Peninsula Laboratories); analytical RPHPLC (apparatus B, 10 μ C_{18} Vydac column (250 mm x 4.6 mm diameter)) 38% B, t_R 21.5 min; t.l.c. R_f 0.38 (S1), R_f 0.61 (S2), R_f 0.75 (S3); amino acid analysis: Asp 1.07, Glu 1.09, Gly 1.01, Arg 3.09, Thr 1.09, Pro 1.06, Tyr 0.86, Val 3.00, Met 1.89; m/z (F.A.B., glycerol matrix) 2099

$(M+H)^+$ [$(M+H)^+$ calcd. 2099.0].

MCH (**1**) *By Cyclisation On The Solid Support.*- To an agitated suspension of the peptidyl-resin of MCH (50 mg) (synthesised as previously described) in DMF (5 cm^3), containing D,L-methionine (3.7 mg, 25 μmol) and D,L-tryptophan (5.1 mg, 25 μmol), was added a solution of iodine (254 mg, 1.0 mmol) in DMF (50 cm^3) under nitrogen. The mixture was agitated for a further 25 min and a cold aqueous solution of ascorbic acid (200 mg in 16 cm^3) was added to the mixture in an ice bath. The resin was isolated by filtration and washed with water-DMF (1:1 v/v, 15 cm^3), DMF (15 cm^3), DCM (15 cm^3) and diethyl ether (30 cm^3) and dried in a stream of nitrogen. The peptidyl-resin was then added to the nitrogen purged cleavage/deprotection mixture of TFA (7.5 cm^3), phenol (400 mg), D,L-methionine (20 mg) and D,L-tryptophan (20 mg) and was left to stand for 2 h at RT. The resin was then removed by filtration and washed with TFA. The combined filtrate and washings were left to stand for a further 5 h at RT, then concentrated under reduced pressure, water (10 cm^3) added, and the resultant solution washed with ether (3 x 10 cm^3). The combined ether layers were back-extracted once with an equal volume of water. The combined aqueous layers were lyophilised to afford a pale brown solid.

An initial purification was carried out by solid phase extraction. This was followed by preparative RPHPLC under the standard conditions to yield the *title compound* (**1**) (1.1 mg, 10%). The purified product was shown to possess no free sulphydryls by the thiol test and was identical to the compound (**1**) prepared by the previous method.

Experimental to Chapter 3

[Phe^{11}] cyclic MCH(5-14) (**49**).- A solution of iodine (389 mg, 1.5 mmol) in an aqueous solution of 80% acetic acid (v/v, 31 cm^3) was added under nitrogen to a stirred solution of (**45**) (10.0 mg, 7.6 μmol), D,L-methionine (5.5 mg, 38 μmol) and 2 *N* hydrochloric acid (10 μl) in 50% acetic acid (1 cm^3). After stirring for 15 h at RT the reaction mixture was added to a stirred suspension of zinc dust (1.34 g, 20.6 mmol) in water (215 cm^3) under nitrogen. After stirring for 18 h the mixture was filtered through a short plug of celite. After washing with water (30 cm^3) the combined filtrate and washings were lyophilised to yield crude material as a mixture of peptide and iodide salt. Isolation of the peptide was achieved by solid phase extraction to yield crude peptide (8.0 mg). The product showed the absence of free thiol. The material was further purified by chromatography on Sephadex LH20 followed by preparative RPHPLC to render the *title compound* (**49**) (1.2 mg, 14%); analytical RPHPLC (apparatus B) 52% B, t_R 28.5 min; t.l.c. R_f 0.38 (S1), R_f 0.64 (S2), R_f 0.79 (S3); amino acid analysis: Gly 1.03, Arg 1.89, Pro 1.01, Val 2.00, Met 0.90, Phe 0.98; m/z (F.A.B., glycerol matrix) 1166 $(M+H)^+$ [$(M+H)^+$ calcd. 1165.6].

[DAla^8] cyclic MCH(5-14) (**50**)

(i) **Preparation of the peptidyl-resin**

Synthesis was carried out using a total of 0.8 g of resin (0.2 m equiv.). For incorporation of the first Fmoc-amino acid, Fmoc-Cys(Trt)-OPfp and DMAP (0.025 molar equivalents) were added and recirculated for 3 h. A second batch was similary employed and recirculated for 15 h followed by a third batch recirculated for 1 h. The quantitative Fmoc determination indicated that a resin coverage of 73% had been obtained. The Fmoc group was cleaved and the synthesis continued as usual, employing Pfp esters, unless otherwise stated. The following Fmoc-amino acids were coupled: Fmoc-Pro (120 min); Fmoc-Arg(Mtr) (Dhbt ester, 25 min); Fmoc-Tyr(*t*-Bu) (25 min); Fmoc-Val (25 min); Fmoc-Arg(Mtr) (Dhbt ester, 25 min). Following cleavage of the Fmoc group the symmetrical anhydride of Fmoc-DAla was prepared in the usual manner, adding a few drops of DMF to achieve dissolution of Fmoc-DAla in DCM. The anhydride formed was seen to precipitate with DCU and the mixture was concentrated under reduced pressure. The resultant suspension was loaded onto the resin in DMF and coupling was complete after the usual 25 min period.

The synthesis was then continued coupling the remaining Fmoc-amino acids: Fmoc-Val (45 min); Fmoc-Met (25 min); Fmoc-Cys(Trt) (25 min).

(ii) **Cyclisation**

The peptidyl-resin (400 mg) was added to the standard cleavage/deprotection mixture for 15 h. An identical work-up and cyclisation procedure to that used for the cyclic MCH(5-14) (**7**) in method (2) (ii) (Chapter 2 Experimental) was employed. Before addition to the air oxidation buffer the crude disulphydryl peptide (35.9 mg) was shown to contain 71% free thiol. The crude cyclic material was subjected to chromatography on Sephadex LH20 to afford the *title compound* (**50**) (22.4 mg, 19%). A portion (6.3 mg) was subjected to preparative RPHPLC to render the pure material (4.3 mg); analytical RPHPLC (apparatus B) 43% B, t_R 24.0 min; t.l.c. R_f 0.26 (S1), R_f 0.40 (S2), R_f 0.61 (S3); amino acid analysis Arg 2.09, DAla 1.08, Pro 1.03, Tyr 0.90, Val 2.00, Met 0.89; m/z (F.A.B., glycerol matrix) 1196 $(M+H)^+$ [$(M+H)^+$ calcd. 1195.6].

[DAla^8,Phe^{11}] cyclic MCH(5-14) (**51**)

(i) **Preparation of the peptidyl-resin**

In a parallel synthesis to that of (**50**) the Phe analogue was prepared, substituting for Tyr^{11} at the appropriate point.

(ii) **Cyclisation**

An identical procedure was employed to that used for the synthesis of (**50**), starting from 400 mg of peptidyl-resin. Chromatography on Sephadex LH20 afforded the *title compound* (**51**) (51.6 mg, 44%). A portion (7.2 mg) was subjected to preparative RPHPLC to furnish the purified product (5.5 mg); analytical RPHPLC (apparatus A) 44% B, t_R 24.5 min; t.l.c. R_f 0.28 (S1), R_f 0.45 (S2), R_f 0.64 (S3); amino acid analysis: Arg 2.06, DAla 0.91, Pro 1.01, Val 2.00, Met 0.92, Phe 0.98; m/z (F.A.B., glycerol matrix) 1180 $(M+H)^+$ [$(M+H)^+$ calcd. 1179.6].

Experimental to Chapter 4

Dimethyl 3-chloropent-2-enedioate[115] **(59)**.- Phosphorus pentachloride (54.00 g, 260 mmol) was added portionwise to diethyl 3-oxo-pentanedioate (47.97 g, 237 mmol) under an atmosphere of nitrogen with stirring. After the addition was complete, the reaction mixture was warmed to 40°C in a water bath for 30 min, during which time a red colouration developed. The reaction mixture was then cooled in an ice bath and poured onto ice (80 cm^3) in an ice-cooled flask. A mixture of water and DCM (1:1 v/v, 100 cm^3) was used to rinse traces of product from the reaction vessel, and the resulting mixture was stirred for 15 min. After separating the two layers, the aqueous phase was extracted with DCM (3 x 80 cm^3) and the combined organic extracts dried (Na_2SO_4) and concentrated under reduced pressure to yield a red oil. This was added to a mixture of concentrated sulphuric acid (13 cm^3) and dry methanol (195 cm^3) and heated under reflux for 18 h. Excess methanol (130 cm^3) was removed by distillation, the residue allowed to cool to RT and then poured into water (80 cm^3). Sodium chloride was added to saturation and the solution extracted with ether (8 x 80 cm^3). The combined ether extracts were washed successively with saturated aqueous $NaHCO_3$ solution (120 cm^3) and saturated aqueous NaCl solution (120 cm^3), dried ($MgSO_4$) and concentrated under reduced pressure to afford a yellow oil. Purification was effected by distillation under reduced pressure to afford the *title compound* **(59)** (26.03 g, 57%) as a colourless liquid, b.p. 70-80°C at 0.1 mmHg, (lit.,[115] b.p. 50-60°C at 0.02 mmHg), t.l.c. R_f 0.36 (petrol-EtOAc 9:1 v/v). The product was determined to be a mixture of stereoisomers (approximately 6:1) by ^1H n.m.r. The spectral data of **(59)** were in accord with published data;[115] υ_{max} (liquid film) 3080 (C=C), 2940, 1720 (CO-O), 1630 (C=C), 1430 cm^{-1}; δ_H ($CDCl_3$) 3.73 (3H, s, $C\underline{H}_3O$-$COCH_2$), 3.75 (3H, s, C=CHCO-$OC\underline{H}_3$), 4.12 (2H, s, CH_3O-$COC\underline{H}_2$), 6.21, 6.28 (1H, 2 x s, stereoisomeric C=CH); δ_C ($CDCl_3$), 41.2 (C=$CH\underline{C}H_2$), 51.6 ($\underline{C}H_3O$-$COCH_2$), 52.3 (C=CHCO-$O\underline{C}H_3$), 121.5 (ClC=$\underline{C}$H), 150.0 (Cl$\underline{C}$=CH), 164.4 (CH_3O-$\underline{C}OCH_2$), 168.1 (C=$CH\underline{C}O$-OCH_3); m/z (C.I.) 193 $(M+1)^+$ (19%), 161 $(M-CH_3O)^+$ (100); [Found: C, 43.7; H, 4.9. $C_7H_9O_4Cl$ requires C, 43.6; H, 4.7%].

Dimethyl penta-2,3-dienedioate[115] **(58)**.- To a stirred solution of dimethyl 3-chloropent-2-enedioate **(59)** (25.19 g, 130 mmol) in dry THF (93 cm^3) at 0°C under a nitrogen atmosphere was added freshly distilled triethylamine (21 cm^3, 151 mmol) portionwise over a 10 min period. The nitrogen inlet/outlet was then replaced by a drying tube ($CaCl_2$) and the mixture stirred for 18 h at 0-5°C. During this time a precipitate of triethylamine hydrochloride formed which was removed by filtration and washed with ether (3 x 25 cm^3). The combined filtrate and washings were washed successively with 0.1 *N* hydrochloric acid (3 x 20 cm^3) and saturated aqueous

NaCl solution (25 cm^3). After drying (Na_2SO_4) the ethereal solution was concentrated under reduced pressure to yield a red brown oil. The oil was purified by distillation under reduced pressure to afford the *title compound* (**58**) (12.62 g, 62%) as a colourless liquid, b.p. 78-80°C at 0.1 mmHg (lit.,[115] b.p. 58°C at 0.02 mmHg), t.l.c. R_f 0.18 (petrol-EtOAc 9:1 v/v). The product became yellow and viscous on standing at RT, due to polymerisation, and was thus stored at -20°C. The spectral data of (**58**) were in accordance with published data;[115] υ_{max} (liquid film) 2960, 1960 (C=C=C), 1710 (CO-O) cm^{-1}; δ_H ($CDCl_3$) 3.78 (6H, s, 2 x CH_3O-CO), 6.07 (2H, s, HC=C=CH); δ_C ($CDCl_3$) 52.3 (2 x $\underline{C}H_3O$-CO), 91.9 ($\underline{C}$=C=$\underline{C}$), 163.5 (CH_3O-$\underline{C}O$), 219.5 (C=$\underline{C}$=C); m/z (E.I.) no $(M)^+$, 125 $(M\text{-}CH_3O)^+$ (77%), 66 (34), 59 (100), (C.I.) 157 $(M+1)^+$ (100), 125 (64).

1,5-Dihydroxypenta-2,3-diene (**57**).- A 1.5 *M* solution of Dibal-H in toluene (17 cm^3, 25.6 mmol) was added portionwise over 1 h to a stirred solution of dimethyl penta-2,3-dienedioate (**58**) (1.00 g, 6.4 mmol) in dry toluene (9.5 cm^3) at 0°C under nitrogen. After 1.5 h of additional stirring at 0-5°C, a saturated aqueous solution of potassium sodium tartrate (23 cm^3) was slowly added to the cooled reaction mixture which was then allowed to warm to RT. The product was isolated by continuous extraction into ether over two consecutive 15 h periods. The combined ethereal isolates were evaporated under reduced pressure to afford an orange oil. Purification of the crude product by column chromatography [silica gel, $CHCl_3$-EtOH (19:1)] gave the *title compound* (**57**) (0.28 g, 44%) as a colourless oil, t.l.c. R_f 0.37 (EtOAc). The product was stored at -20°C as polymerisation occurred on standing at RT; υ_{max} (liquid film) 3600-3100 (OH), 2920, 2860, 1970 (C=C=C) cm^{-1}; δ_H ($CDCl_3$) 2.39 (2H, bs, OH), 4.16 (4H, t, J = 4.2 Hz, 2 x $C\underline{H}_2OH$), 5.51 (2H, t, J = 4.2 Hz, HC=C=CH); δ_C ($CDCl_3$) 60.1 (2 x CH_2OH), 94.7 ($\underline{C}$=C=$\underline{C}$), 202.5 (C=$\underline{C}$=C); m/z (E.I.) no $(M)^+$, 82 $(M\text{-}H_2O)^+$ (99%), 55 (100), (C.I.) 101 $(M+1)^+$ (8), 100 (69), 83 $(M+1\text{-}H_2O)^+$ (100), 56 (26).

5-(t-Butyldiphenylsilyloxy)penta-2,3-diene-1-ol (**60**).- To a stirred solution of 1,5-dihydroxypenta-2,3-diene (**57**) (174 mg, 1.7 mmol) in dry DCM (10 cm^3) containing imidazole (118 mg, 1.7 mmol) under a nitrogen atmosphere was added *t*-butyldiphenylsilyl chloride (479 mg, 1.7 mmol) dropwise at RT. After 2 h the reaction mixture was filtered through a celite pad and evaporated under reduced pressure to afford the crude silyl ether which was purified by column chromatography [silica gel, petrol-EtOAc (10:1)] to yield the *title compound* (**60**) (304 mg, 52%) as a colourless oil, t.l.c. R_f 0.41 (petrol-EtOAc 4:1 v/v); υ_{max} (liquid film) 3600-3100 (OH), 3060 and 3020 (Ar), 2920, 2840, 1950 (C=C=C), 1100 and 820 (SiOC) cm^{-1}; δ_H ($CDCl_3$) 1.05 (9H, s, $C(CH_3)_3$), 1.54 (1H, bs, OH), 4.08 (2H, dd, J = 8.6, 3.2 Hz,

$HOC\underline{H}_2$), 4.25 (2H, dd, J = 8.8, 3.1 Hz, CH_2OSi), 5.41 (2H, c, HC=C=CH), 7.35-7.73 (10H, c, H-aromatic), δ_C ($CDCl_3$) 19.3 ($\underline{C}(CH_3)_3$), 26.9 ($C(\underline{C}H_3)_3$), 60.5 ($HOCH_2$), 61.9 (CH_2OSi), 93.6 (HC=C=CH-$\underline{C}H_2OSi$), 94.4 (HOH_2C-H$\underline{C}$=C=CH), 127.7, 129.7, 133.7, 135.6 (C-aromatic), 202.8 (C=$\underline{C}$=C); m/z (C.I.) 339 $(M+1)^+$ (5%), 321 $(M+1-H_2O)^+$ (83), 229 (58), 199 (100), 143 (75); [Found: C, 74.4; H, 7.9. $C_{21}H_{20}O_2Si$ requires C, 74.5; H, 7.8%].

5-(t-Butyldiphenylsilyloxy)penta-2,3-diene-1-oxy-methylsulphone (**61**).-
Triethylamine (75 mg, 0.7 mmol) was added dropwise to a stirred solution of (**60**) (248 g, 0.7 mmol) and methanesulphonyl chloride (84 mg, 0.7 mmol) in dry DCM (5 cm^3) at 0°C under nitrogen. After stirring for a further 2 h at 0-5°C the solution was poured into a mixture of 2 *N* hydrochloric acid and ice (2:1 v/v, 5 cm^3). The organic layer was isolated and the aqueous layer extracted further with DCM (2 x 5 cm^3). The combined organic layers were washed with saturated aqueous $NaHCO_3$ solution (2 x 10 cm^3) and saturated aqueous NaCl solution (10 cm^3), dried (Na_2SO_4) and concentrated under reduced pressure to afford the mesylate (**61**) (224 mg, 77%) as a pale yellow oil. Storage at -20°C was employed to prevent decomposition. The compound was to be used without further purification as decomposition occurred during column chromatography; υ_{max} (liquid film) 3040 and 3020 (Ar), 2940, 2840, 1960 (C=C=C), 1340 and 1160 (SO_2O), 1090 and 820 (SiOC) cm^{-1}; δ_H ($CDCl_3$) 1.05 (9H, s, $C(CH_3)_3$), 2.97 (3H, s, CH_3SO_2O), 4.26 (2H, dd, J = 8.6, 2.9 Hz, CH_2OSi), 4.66 (2H, dd, J = 9.3, 2.0 Hz, SO_2OCH_2), 5.42 (2H, c, HC=C=CH), 7.37-7.69 (10H, c, H-aromatic); δ_C ($CDCl_3$) 19.2 ($\underline{C}(CH_3)_3$), 26.8 ($C(\underline{C}H_3)_3$), 38.3 (CH_3OSO_2), 61.6 (CH_2OSi), 67.8 (SO_2OCH_2), 87.7 (SO_2OCH_2-$\underline{C}$H=C=CH), 94.4 (HC=C=$\underline{C}$H-CH_2OSi),127.7, 129.8, 133.7, 135.5 (C-aromatic), 202.5 (C=$\underline{C}$=C); m/z (C.I., NH_3) 434 $(M+NH_4)^+$ (98%), 417 $(M+1)^+$ (21), 338 $(M+1-SO_2CH_3)^+$ (13), 321 $(M-OSO_2CH_3)^+$ (52), 143 (74).

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APPENDIX

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Melanin Concentrating Hormone; Molecular Modelling and Experimental Analysis of Conformation

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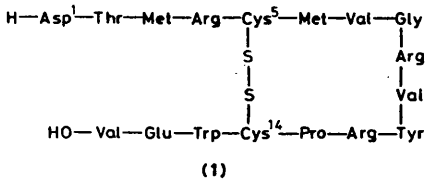
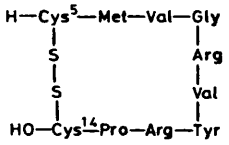
Molecular dynamics analyses and associated energy minimizations for the peptide hormone MCH and the related cyclic decapeptide MCH(5—14) suggest that it may access two major families of conformations; experimental support comes from n.m.r. studies of MCH(5—14).

Melanin concentrating hormone (MCH) (1) is a neuropeptide produced in the hypothalamus. In teleosts it serves to concentrate melanin within the pigment cells of the skin.[1—3] It also induces melanosome dispersion within tetrapod melanophores.[4] MCH also acts as a potent pituitary hormone, inhibiting the release of ACTH in mammals,[5] and stimulating growth hormone release in rats.[6]

As the first phase in elucidating the shape and charge characteristics of MCH receptors we have performed theoretical analyses of the conformational features of the cyclic decapeptide, MCH(5—14) (2), and of the intact peptide (1). Additionally, we have synthesized these two peptides,[7] and a range of fragments, for biological assay and for n.m.r. analysis of conformation. In this communication we report the results of the theoretical predictions from molecular dynamics analyses, together with structural information obtained from n.m.r. spectroscopy.



(1)



(2)

Molecular dynamics methods have been employed recently to reveal accessible conformations and to characterize the dynamic conformational transitions of proteins and peptide hormones.[8] We therefore applied molecular dynamics simulations and energy minimization procedures using a valence force field software package for MCH and for the cyclic decapeptide MCH(5—14). In the valence force field,[9] the potential energy of a molecular system is represented as a sum of the internal or valence degrees of freedom and the interatomic distances. The analytical expression of the potential energy[10] includes (a) strain energies arising from deformations of internal co-ordinates (like bond length, bond angle, and torsion angle) and 'cross terms' caused by coupling between deformations of two or more internals and (b)

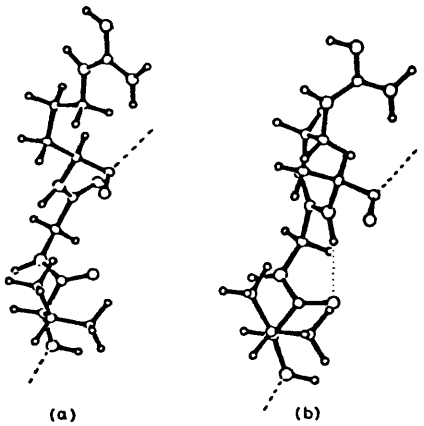


Figure 1. Region of the peptide around Val⁷-Gly⁸-Arg⁹ when Gly is in conformations which are (a) an α-helical and (b) a γ turn.

Table 1. Chemical shift assignments for MCH(5—14) in DMSO solution at 293 K.

	NH	α	β	γ	δ	ε
5 Cys		4.54	~3.0			
6 Met	—[b]	4.52	1.90, 2.09	2.96, 2.56		
7 Val	7.73	4.12	1.90	0.83		
8 Gly	8.41	3.90, 3.65				
9 Arg[a]	7.83	4.55	1.78, 1.55	2.40	~3.1	9.23 or 8.70
10 Val	8.10	4.10	1.92	0.70		
11 Tyr	8.16	4.52	2.90, 2.78			
12 Arg[a]	8.12	4.17	1.77, 1.52	2.43	~3.2	9.23 or 8.70
13 Pro		4.33	2.02, 1.97	2.40	~3.5	
14 Cys	8.08	3.98	2.92, 3.24			

[a] Assignment uncertain. [b] Cross-peak absent.

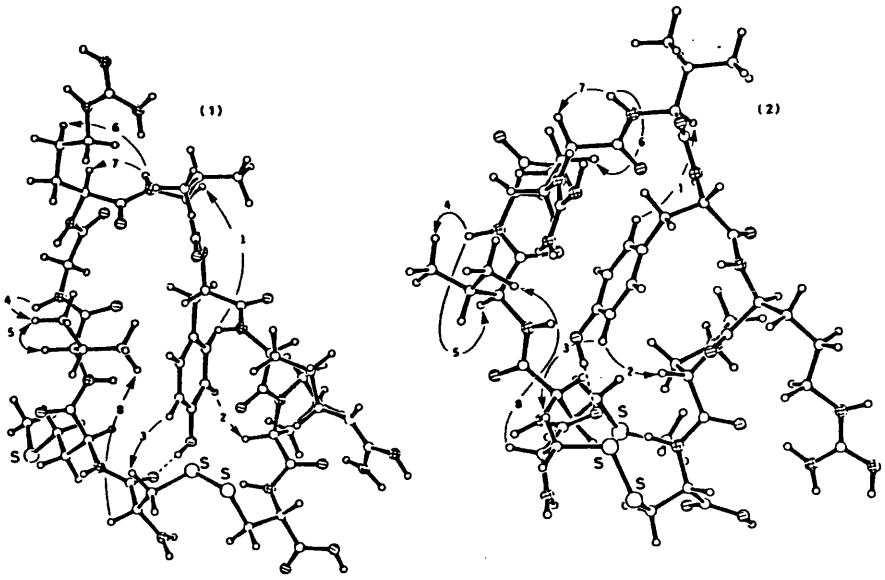


Figure 2. Typical conformations of MCH(5—14) showing n.O.e.s.

interaction energies which are a result of the exchange repulsion, dispersion, and coulomb interactions between the non-bonded atoms. The hydrogen bond energy term is represented as a general non-bonded interaction but with specific parameters. A fixed dielectric with a dielectric constant of 1 was used in the calculations. The parameters included in the various energy terms have been determined by fitting experimental crystal structure data, sublimation energies, molecular dipole moments, vibrational spectra, and strain energies of small organic compounds. *Ab initio* molecular orbital calculations have also been used to provide information on charge distributions, energy barriers, and coupling terms.[11] The effect of solvent has not been modelled. Thus, given the potential energy of a molecular system, the force exerted on each atom by all the other atoms in the system is defined and consequently the equations of motion can be solved. This yields a detailed description of the dynamical behaviour of the system. The conformations accessed at different instances in the dynamics trajectory can then be minimised and studied. Here main chain torsion angles of energy minimized conformations were analysed at picosecond intervals along a dynamics trajectory of 50 picoseconds. Each conformation was minimized to a maximum first derivative of less than 0.05 kcal/Å (cal = 4.184 J) and (ϕ/ψ) angles analysed for all amino acids.

The initial conformation of MCH was built on an E&S PS330 picture system, using the INSIGHT package. The residues in the two tails (*i.e.* residues 1—4 and 15—17) were then removed to generate the initial structure of cyclic MCH(5—14). This structure was partially minimised (to a maximum first derivative of 0.5 kcal mol^{-1} Å^{-1}) to relieve excessive strain and eliminate unrealistic motion. Initial random velocities consistent with a Maxwell–Boltzmann distribution for an average temperature of 300 K were assigned to each of the atoms. Molecular dynamics simulations with a time step of 10^{-15} s were performed for a total time period of 50 picoseconds (50×10^{-12} s). The results of the simulations showed some interesting structural features. Firstly, the maximum conformational change occurred in the Gly^{8}–Arg^{9} region of the peptide. Transitions between two conformational states persisted throughout the simulations. The Gly^{8} residue existed in either a γ turn (−80,50) or an α-helical (−70,−40) type of conformation (Figure 1). The Arg^{9} residue also existed in two conformations, with (ϕ,ψ) values of (−155,100) and (−80,100). In other words the ϕ of Gly^{8} and the ψ of Arg^{9} do not undergo much change, whereas the ψ of Gly^{8} and the ϕ of Arg^{9} undergo concerted transitions. Whenever the near type I Gly^{8}–Arg^{9} β-turn occurs the Arg^{9}–Val^{10} forms a near type II β-turn. The other mobile residue in this region is Val^{7}. Met^{6} and Val^{7} exist mainly in a left-handed helix or in a β-sheet structure.

Regions of steric constraint emerged from the simulation. Val^{10} to Cys^{14} showed little change, Tyr^{11} to Cys^{14} being in a stable helix with (ϕ,ψ) around (−80,110) (near the polyproline helix). Interestingly, Pro^{13} which is known to be an initiator of β-turns does not participate in any of these in the simulation. A major conformational feature which persisted throughout the analyses was a transannular hydrogen bond from Tyr^{11} to Cys^{5} or Tyr^{11} to Met^{6}. The preference for this conformationally constraining feature was most significant. Further features leading to relative rigidity in this section of the molecule included the stable +90° disulphide conformation and a stable hydrogen bond between the ϵ-NH of Arg^{9} and the NH of Val^{10}.

Additional molecular dynamics simulations were performed on MCH(5—14) starting from a conformation which was not characterised by the cross-ring Tyr^{11} to Cys^{5} hydrogen bond. However, it was found that the conformational features of the minima accessed during the new simulations were very similar to those found previously. In particular, the Tyr^{11} to Cys^{5} transannular hydrogen bond was once again found to be present in all the minimum energy conformations. It was further apparent that the overall backbone conformations of MCH(5—14) and MCH were closely similar, and that the

Table 2. Inter-residue 1D nuclear Overhauser enhancements for MCH(5—14) in DMSO solution.

			Enhancement	Interatomic distance/Å[a] Conf. 1	Conf. 2
1	$C^{\delta}_{11}H$	$C^{\alpha}_{10}H$	1	6.6	3.9
2	$C^{\epsilon}_{11}H$	$C^{\alpha}_{13}H$	1	3.1	2.9
3	$C^{\epsilon}_{11}H$	C^{β}_{5} or $C^{\beta}_{14}H$	0.5	4.1[b]	3.2[b]
4	N_8H	$C^{\alpha}_{7}H$	3	3.5	2.1
5	N_8H	$C^{\alpha}_{7}H$	15	2.1	2.4
5[a]	$C^{\alpha}_{7}H$	N_8H	20	2.1	2.4
6	$N_{10}H$	$C^{\alpha}_{9}H$	4	4.0	2.7
7	$N_{10}H$	$C^{\alpha}_{9}H$	6	2.1	2.3
8	$C^{\alpha}_{5}H$	$C^{\alpha}_{7}H$	2	6.0	5.6

[a] The interatomic distances are obtained from conformations observed in molecular dynamics simulations and where more than one hydrogen is designated represents the smallest value. [b] N.m.r. assignments ambiguous. Distances based on $C_{14}H$.

mobile side chains did not induce significant changes. MCH(5—14) retains significant activity relative to MCH.[1—4]

Independent n.m.r. analyses were performed on MCH(5—14) at 400 MHz both in water and in dimethyl sulphoxide (DMSO). Chemical shift assignments were made (Table 1) from COSY, long range COSY, and NOESY measurements. The proton signals for MCH(5—14) were highly dispersed in relation to linear MCH(5—14), the α-protons of Gly[8], for example, being strongly differentiated and complete resolution of all NH and Cα-H protons was achieved, consistent with greater conformational restraints. Variable temperature studies of backbone amide and arginine ε-NH protons were performed. In H_2O the shallowest slope was shown by the Arg[9] guanidinium protons, the remaining coefficients being large and therefore not strongly internally hydrogen bonded. In DMSO the guanidinium NH units had the largest temperature coefficients, with uniformly low values for the amide NH units with the exception of Cys[14] and Gly[8]. This may be regarded as indicating more rigid, hydrogen bonded conformations existing in DMSO. Conformationally significant n.O.e. data were obtained only in DMSO. The most interesting of these involved Tyr[11], which, by 1D differential n.O.e. showed connectivities from Tyr[11]-CεH to Pro[13]-CαH (1.5%), Tyr[11]-CδH to Val[10]-CαH (1%), and from Tyr[11]-CεH to one of the Cys Cβ-H protons (0.5%). These small, but reproducible, effects suggest a conformation in which Tyr[11] occupies a transannularly hydrogen bonded conformation. (Tyr[11]OH was not detected.) Other inter-residue n.O.e. effects (Table 2) were also in accord with the two conformations shown in Figure 2 which had previously been predicted by molecular dynamics and are typical of the two different families of accessible, minimized conformations obtained from each of the dynamics trajectories. The great majority of these minimized conformations satisfy the spatial requirements for either six of the eight or seven of the eight observed n.O.e. effects depending upon the family. *All* accessible minimized conformations feature the transannular tyrosine hydrogen bond.

Independent theoretical and experimental studies are thus mutually compatible in these preliminary studies. Constraining features and regions of conformational flexibility have been defined. Further synthetic studies designed to lock one or other conformation, together with n.m.r. analysis of conformation and assessment of biological activity are in progress and will be reported.

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